AMENDMENTS TO THE DRAWINGS:

The attached sheets of drawings are new to the application but are fully described and thus, supported, in the specification at pages 13-17. Applicants request entry of the drawings.

REMARKS

Claims 25, 34, 36 and 37 are pending in this application. Claims 1-24, 26-33, 35, and 38-50 are canceled without prejudice to Applicants' right to pursue the subject matter of these claims in a related application.

Election/Restriction

In response to the restriction requirement, Applicants have amended the claims to specifically recite PD098059 as the second compound.

Drawings

In accordance with the recommendation of the Examiner's supervisor, Deborah Reynolds, Applicants submitted DRAFT Figures 1-8 (9 sheets) in the Reply to Office Action filed February 13, 2004, and requested permission to amend the application to include the referenced drawings. Because the Examiner has not acknowledged this request, Applicants now submit a formal amendment to the application to include Figures 1-8. The drawings are described in the specification in great detail at page 13, line 24 to page 17, line 5. Thus, amendment of the application to include the actual drawings themselves does not add new matter.

Claim Objections

The claims as amended overcome the Examiner's objections.

35 U.S.C. § 112, first paragraph

The claims as amended are fully supported by the application as filed and comply with the written description and enablement requirements of 35 U.S.C. §112,

first paragraph. Issues raised by the Examiner in connection with canceled claims are considered moot.

The Examiner suggests that the "limitation of 'dissociating the cells' and 'maintaining the dissociated cells' in claims 34 and 37 are new matter and lack written description." (Office Action at p. 11.) Applicants respectfully submit that both claims 34 and 37 are fully supported by the application as filed.

Original claim 35 recited both steps of "dissociating the cells" and "maintaining the dissociated cells." Thus, no new matter has been added to these claims. Further support for the recited steps can be found in Example 2 of the specification (page 27, lines 8-12). Example 2 states that epiblasts were isolated and then maintained in culture in the presence of P098059. The epiblasts were then dissociated and the dissociated cells were maintained in culture in the presence of PD098059. Consequently, Example 2 provides written description support for claims 34 and 37.

The Examiner contends that "the limitation of developing an embryo in vitro (claim 37) is new matter." (Office Action at p. 11.) Because this language was part of the original claim, it cannot be considered new matter.

Claims 25, 34, 36 and 37 stand rejected as allegedly failing to comply with the enablement requirement of 35 U.S.C. §112, first paragraph. (Office Action at p. 12.) The Examiner contends that the claims are not enable because the specification does not teach how to generate the genetically altered ES cells used as the starting material in the assays described.

Applicants previously argued that the constructs used to exemplify the methods of the of the invention are of a type known in the art. The Examiner correctly notes that

U.S. Patent 6,150,169, relied upon by applicants was not available to the public until two years after the effective filing date of the present application. However, WO 94/24301, the international application on which that US patent was based, was published on October 27, 1994, before the filing of the present application. Sufficient detail of how to make constructs of the type used in the claimed invention is provided in WO 94/24301 and in Mountford et al., previously submitted.

The application as filed explains, e.g., that beta-galactosidase was expressed from the Oct 4 locus – see page 18, first 2 lines. This makes clear that the Oct 4 promoter drives expression of the beta- galactosidase transgene. This disclosure, in combination with Mountford et al. and WO 94/24301, is sufficient for a person of skill in the art to make the construct.

It should be noted that the examples were carried out to confirm to the inventors' satisfaction that LIF and PD 098059 enhanced self-renewal of ES cells and that this observation was not attributable to other factors. The invention claims that a combination of LIF and PD098059 can be used to culture ES cells with increased self-renewal of those ES cells. To carry out the invention a skilled person need only culture ES cells in LIF and PD098059. The skilled person need not repeat the proof of principle work carried out by the inventors which the inventors deemed necessary before the inventors were prepared to declare their invention to the public.

Moreover, it is not necessary to use genetically modified ES cells for the invention to work. As noted above, these constructs were used to test the principles of the invention and confirm the effects were attributable to LIF and PD098059 rather than

to other factors. The invention will in practice be carried out on genetically altered ES cells but also, and more preferably, on ES cells that have not been genetically altered.

Applicants draw the Examiner's attention to the following references which identify ZIN40, DO27 and IOUD2 cells as ES cells:

ZIN40 - Charriere et al., Abstract from NCBI, sample GSM26334

IOUD2 - Abstract from Physiology Image Gallery

DO27 - Niwa et al., Genes and Development, 12(13): 2048-2060 (1998).

Applicants respectfully submit that the amended claims are fully enabled and request that the rejection under 35 U.S.C. § 112, first paragraph, be withdrawn.

35 U.S.C. § 112, second paragraph

Applicants submit that the amended claims obviate the rejections under 35 U.S.C. § 112, second paragraph.

35 U.S.C. § 102

Claim 25 stands rejected under 35 U.S.C. § 102(a) as allegedly anticipated by Niwa et al. As amended, claim 25 requires that the culture medium is free of ES cells. This claim is not anticipated by Niwa et al., which does not disclose medium that is free of ES cells but also contains both LIF and PD098059. Support for the amendment to claim 25 is found in the specification at page 9, lines 1-3, which describes ES cells in the culture medium. Prior to the addition of the ES cells, this culture medium is free of ES cells. Accordingly, Applicants submit that claims 25, 34, 36, and 37 is free of the art.

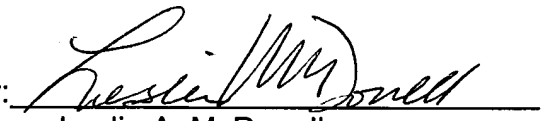
In view of the foregoing amendments and remarks, Applicants respectfully request reconsideration and reexamination of this application and the timely allowance of the pending claims.

Please grant any extensions of time required to enter this response and charge any additional required fees to deposit account 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: October 18, 2004

By: 
Leslie A. McDonell
Reg. No. 34,872

Attachments:

Formal Drawings (9 sheets)
Charriere et al., Abstract from NCBI, sample GSM26334
Abstract from Physiology Image Gallery
Niwa et al., Genes and Development, 12(13): 2048-2060 (1998)
WO 94/24301

Options: Scope: Self Format: HTML Amount: Quick GEO accession: GSM26334

Sample GSM26334 Query DataSets for GSM26334

Status	Public on Sep 1 2004
Title	non-differentiated ESC #1
Type	single channel
Organism	Mus musculus
Target source	ZIN40 ES cell line
Description	ZIN40 were cultered in low density to avoid confluence state. The cells were cultured in GMEM + 10% FCS medium + LIF to maintain totipotence. RNA extraction was performed with TRIPURE reagents and protocol. RNA were trated with DNASE before using them for the hybridization. cDNA were labeled with dCTP33P. Hybridization was performed according to the manufacturer protocol (Resgen). Quantification was performed with Imagene software.
Keyword	Mus musculus, Embryonic stem cell, ZIN40.
Author	Charriere G , Casteilla L , Arnaud E , Cousin B , Andre M , Penicaud L
Submission date	Jun 30 2004
Submitter name	Charriere, Guillaume
Submitter email	guillaume.charriere@toulouse.inserm.fr
Submitter institute	IFR31
Submitter laboratory	UMR 5018 UPS-CNRS
Submitter department	
Submitter city	Toulouse, 31400 France
Submitter phone	(33) 5 61 32 34 95
Submitter web link	
Platform id	GPL1285
Series id	GSE1536

Data table header descriptions

ID_REF

VALUE addition all pixels intensities for each spots (Signal total calculated by Imagene software)

Data table

ID_REF	VALUE
1	426801.0
2	424611.0
3	87623.0
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9	53431.0
10	45564.0
11	50661.0
12	57362.0
13	65676.0
14	71441.0
15	53730.0
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17	57310.0
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19	51789.0
20	44414.0

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Embryonic Stem Cells

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DUAL NUCLEAR AND SSEA STAINING OF IOUD2 CELLS PASSAGE 14, MAGNIFICATION 40X SINGLE SLICE

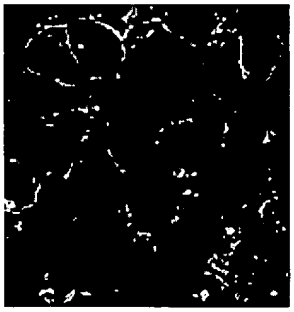
Work by Sheila Faherty

CCE CELLS PASSAGE No. 23 STAINED FOR SSEA MAGNIFICATION 40X ZOOM 1.6 MAXIMUM PROJECTION

Work by Sheila Faherty

IOUD2 CELLS PASSAGE 14, STAINED FOR SSEA MAGNIFICATION 40X ZOOM 2.0

Work by Sheila Faherty



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Vol. 12, No. 13, pp. 2048-2060, July 1, 1998

RESEARCH PAPER

Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3

Hitoshi Niwa,[1,2] Tom Burdon,[1] Ian Chambers, and Austin Smith[3]

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The propagation of embryonic stem (ES) cells in an undifferentiated pluripotent state is dependent on leukemia inhibitory factor (LIF) or related cytokines. These factors act through receptor complexes containing the signal transducer gp130. The downstream mechanisms that lead to ES cell self-renewal have not been delineated, however. In this study, chimeric receptors were introduced into ES cells. Biochemical and functional studies of transfected cells demonstrated a requirement for engagement and activation of the latent trancription factor STAT3. Detailed mutational analyses unexpectedly revealed that the four STAT3 docking sites in gp130 are not functionally equivalent. The role of STAT3 was then investigated using the dominant interfering mutant, STAT3F. ES cells that expressed this molecule constitutively could not be isolated. An episomal supertransfection strategy was therefore used to enable the consequences of STAT3F expression to be examined. In addition, an inducible STAT3F transgene was generated. In both cases, expression of STAT3F in ES cells growing in the presence of LIF specifically abrogated self-renewal and promoted differentiation. These complementary approaches establish that STAT3 plays a central role in the maintenance of the pluripotential stem cell phenotype. This contrasts with the involvement of STAT3 in the induction of differentiation in somatic cell types. Cell type-specific interpretation of STAT3 activation thus appears to be pivotal to the diverse developmental effects of the LIF family of cytokines. Identification of STAT3 as a key transcriptional determinant of ES cell self-renewal represents a first step in the molecular characterization of pluripotency.

[*Key Words: Leukemia inhibitory factor (LIF); cytokine receptor; signaling; ES cells; tetracycline; episome*]

Introduction

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Embryonic stem (ES) cells are pluripotent cell lines derived by culture of preimplantation mouse embryos (Evans and Kaufman 1981; Martin 1981; Brook and Gardner 1997). At present, ES cells are the only nontransformed mammalian stem cells that can be continuously propagated in vitro. ES cell self-renewal is sustained by the cytokine leukemia inhibitory factor (LIF) (Smith and Hooper 1987; Smith et al. 1988; Williams et al. 1988). The effect of LIF is to inhibit differentiation and support proliferation of undifferentiated stem cells. However, the mechanisms underlying the maintenance of pluripotency during proliferative expansion remain elusive. We are attempting to define those signaling processes downstream of the LIF receptor complex that direct ES cell self-renewal. Elucidation of these principles will provide a molecular model for stem cell regulation in mammals. Insights provided by such a model should also be directly applicable to the extension of ES cell technology to nonmouse species.

The actions of LIF are mediated via heterodimerization of two members of the class I cytokine receptors, the low-affinity LIF receptor (LIF-R) and gp130 (Gearing et al. 1991; Gearing and Bruce 1992; Davis et al. 1993). The LIF-related cytokines, oncostatin M (OSM), cardiotrophin (CT-1), and ciliary neurotrophic factor (CNTF), act through the same receptor complex (in the case of CNTF, additionally including the CNTF-Rα subunit) and can similarly sustain ES cell self-renewal (Conover et al. 1993; Rose et al. 1994; Wolf et al. 1994; Yoshida et al. 1994; Pennica et al. 1995b). Furthermore, ES cells can also be derived and maintained using a combination of interleukin-6 and soluble interleukin-6 receptor (IL-6/sIL-6R) (Nichols et al. 1994; Yoshida et al. 1994). In this case, signaling is initiated via formation of gp130 homodimers without involvement of LIF-R (Murakami et al. 1993; Yoshida et al. 1994). Signals that emanate from gp130 are therefore sufficient for self-renewal.

gp130 mediates cellular responses to IL-6 and IL-11 in addition to the LIF-related cytokines (Kishimoto et al. 1994). All of these factors exert pleiotropic effects on diverse cell types in vitro and in vivo. In addition to ES cell self-renewal, stimulation of gp130 receptor complexes causes differentiation and growth inhibition in M1 myeloid leukemic cells (Tomida et al. 1984), induction of acute

phase gene expression in hepatocytes (Baumann and Wong 1989), cholinergic differentiation of sympathetic neurons (Yamamori et al. 1989), survival of motor neurons (Li et al. 1995), proliferative and hypertrophic responses in cardiomyocytes (Hirota et al. 1995; Pennica et al. 1995a; Yoshida et al. 1996), and astrocyte differentiation of neuroepithelial progenitors (Bonni et al. 1997; Koblar et al. 1998).

Signaling processes downstream of gp130 are complex and are not yet fully characterized. Ligand-induced dimerization of the receptors (Davis et al. 1993; Murakami et al. 1993) leads to phosphorylation and activation of associated JAK tyrosine kinases (Narazaki et al. 1994; Stahl et al. 1994). The cytoplasmic domain of gp130 contains several tyrosine residues that are phosphorylated by the activated JAKs. These phosphotyrosine residues then interact with SH2 domain containing proteins that in turn themselves become targets for JAKs and possibly other nonreceptor tyrosine kinases. Consequences include activation of the Ras mitogen-activated protein (MAP) kinase (ERK) signaling cascade (Boulton et al. 1994; Yin and Yang 1994; Sheng et al. 1997) and of the STAT factors STAT1 and STAT3 (Lutticken et al. 1994; Stahl et al. 1995). STAT proteins are latent transcription factors that upon phosphorylation, dimerize and translocate to the nucleus where they activate target gene transcription (for review, see Ihle 1996). In myeloid leukemic M1 cells, activation of STAT3 appears to be the main effector of the differentiation response to IL-6 or LIF (Minami et al. 1996; Nakajima et al. 1996). STAT3 activation has also been adduced to mediate CNTF or LIF-induced differentiation of neuroepithelial precursors into astrocytes (Bonni et al. 1997).

In this study we have examined the receptor requirements for self-renewal signaling in ES cells and determined a critical contribution of STAT3 activation. In contrast to its role in somatic cells, activated STAT3 acts to suppress differentiation in ES cells.

Results

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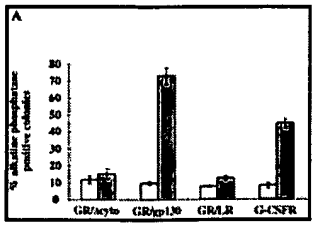
Granulocyte colony-stimulating factor receptor can signal ES cell self-renewal

Granulocyte colony-stimulating factor receptor (G-CSF-R) is a class I cytokine receptor that is evolutionarily related to gp130 and LIF-R (Gearing et al. 1991; Chambers et al. 1997). G-CSF-R is not present in ES cells. To begin delineating the signaling requirements for ES cell propagation, the capacity of these related receptors to sustain self-renewal was compared directly.

G-CSF-R undergoes ligand-induced homodimerization to produce an active signaling complex. G-CSF responsiveness can therefore be conferred on cytoplasmic domains of heterologous receptors through construction of appropriate fusions. cDNAs encoding full-length G-CSF-R cDNA and fusions between the extracellular portion of G-CSF-R and the transmembrane and cytoplasmic region of gp130 or LIF-R were cloned into the expression vector pPCAGIZ. Plasmids were introduced into LIF-R-deficient ES cells to eliminate the contribution of autocrine LIF signaling (Rathjen et al. 1990) from subsequent analyses. In this and all other experiments, ES cells were grown without feeder layers (Smith 1991). Transfectants were selected and expanded in the presence of IL-6/sIL-6R, acting through endogenous gp130, to avoid any selective pressure for adaptation to the introduced receptor.

Stable transfectants were then plated at clonal density in the absence of cytokine or presence of IL-6/sIL-6R or G-CSF. The number of stem cell colonies generated was scored after 6 days. The data in Figure 1A show that the G-CSF-R/gp130 chimeric receptor sustained stem cell propagation in response to G-CSF. This result is anticipated from previous findings on the capacity of gp130 homodimers to signal self-renewal (Yoshida et al. 1994). The G-CSF-R/LIF-R chimera did not support formation of stem cell colonies despite higher levels of cell surface expression measured by radioligand binding (not shown). This is in line with previous reports that homodimerization of the LIF-R cytoplasmic domain results in quantitatively (Baumann et al. 1994a; Stahl et al. 1995) and qualitatively (Stahl et al. 1995) diminished activation of downstream pathways compared with LIF-R/gp130 heterodimerization or gp130 homodimerization. However, ES cells transfected with G-CSF-R did form stem cell colonies in response to G-CSF-R though with lower efficiency than cells expressing the G-CSF-R/gp130 chimera. This somewhat surprising finding corroborates similar data reported recently (Starr et al. 1997). Propagation of the G-CSF-R transfectants remained factor dependent, and the cells differentiated normally when deprived of cytokine.



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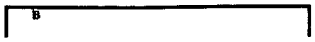
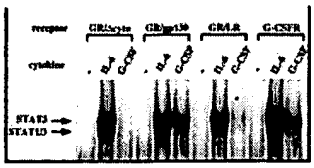


Figure 1. ES cell self-renewal and induction of STAT DNA-binding activity mediated by G-CSF-R wild-type, truncated, and chimeric cytokine receptors. (*A*) Efficiency of clonal stem cell renewal in response to G-CSF measured by formation of alkaline phosphatase-positive colonies. (Light gray bars) −G-CSF; (dark gray bars) +G-CSF. Data are mean ± S.E.M. of triplicate determinations on single representative clones normalized to response to IL-6/sIL-6R. (*B*) Induction of STAT DNA binding by IL-6/sIL-6R and G-CSF determined by electophoretic mobility-shift assay. Cells were untreated or stimulated for 30 min with IL-6/sIL-6R or G-CSF (30 ng/ml). Nuclear extracts were prepared and assayed for SIE binding. Note the absence of detectable STAT1/STAT3 heterodimer complex on stimulation of full-length G-CSF-R.



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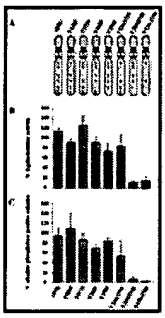
The finding that G-CSF-R is competent to maintain the stem cell phenotype suggests that the signaling interactions essential for ES cell self-renewal are preserved between gp130 and G-CSF-R. Conserved features in the intracellular domains of these two receptors are not readily identifiable because of extensive sequence divergence. However, G-CSF-R contains a putative STAT binding site and is thought to signal primarily through activation of STAT3 (Shimozaki et al. 1997). Electrophoretic mobility-shift assays were performed to determine the induction of nuclear STAT DNA-binding activity by G-CSF in the various ES cell transfectants. Significant STAT3 activation was evident in ES cells transfected with expression vectors for the G-CSF-R/gp130 chimera or the full-length G-CSF-R. In contrast, ES cells expressing the G-CSF-R/LIF-R chimera showed only weak induction of STAT3 DNA-binding activity in response to G-CSF (Fig. 1B). Antibody supershift experiments (not shown) confirmed that the DNA-binding complex consisted predominantly of STAT3 homodimers with a minor component of STAT3/STAT1 heterodimer as described previously in ES cells and other systems (Hocke et al. 1995; Stahl et al. 1995; Starr et al. 1997). These observations pointed to a potentially critical role for STAT3 activation in mediation of the self-renewal signal.

STAT3 docking sites on gp130 are required to signal ES cell self-renewal

The cytoplasmic domain of mouse gp130 contains seven tyrosine residues. Four of these have been identified as phosphorylation-dependent sites of interaction with STAT3 (Stahl et al. 1995). Substitution of these tyrosine residues with phenylalanine in the context of the G-CSF-R/gp130 chimera was therefore used to determine their significance for self-renewal signaling. The modified chimeric receptor expression constructs were introduced into DO27 ES cells. These cells are LIF-deficient because of targeted deletion of both gene copies and, in addition, carry a β-galactosidase reporter integrated into one allele of the *Oct-4* gene (C. Dani, I. Chambers, S. Johnstone, M. Robertson, B. Ebrahimi-Chahardahcherik, M. Saito, T. Taga, M. Li, T. Burdon, J. Nichols, and A.G. Smith, in prep.). This reporter is expressed only in undifferentiated ES cells (Mountford et al. 1994). Self-renewal was assayed both by measuring β-galactosidase activity in medium density cultures (Fig. 2B) and by scoring formation of alkaline phosphatase positive colonies at clonal density (Fig. 2C). Three independent transfectant clones were analyzed for each receptor. The data summarized in Figure 2 demonstrate that the presence of STAT3 docking sites is essential for stem cell propagation.



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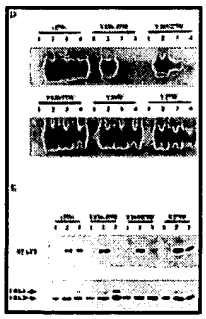

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Figure 2. Effect of mutating STAT3 interaction sites in gp130 on ES cell self-renewal and induction of STAT3 DNA-binding activity. (*A*) Schematic of the various chimeric receptors indicating the tyrosine-phenylalanine substitutions introduced into the wild-type (278) gp130 cytoplasmic domain. Numbering commences with the first residue of the 278-amino-acid intracellular domain of mouse gp130. The phenylalanine (F) for tyrosine (Y) substitutions in the four STAT3 docking sites are indicated. The additional three tyrosines do not interact with STAT3 (Stahl et al. 1995). (*B*) Stem cell renewal mediated by chimeric receptors in response to G-CSF measured by β-galactosidase expression from the *Oct-4* locus. Data are mean ± S.E.M. for duplicate determinations on three independent clones normalized relative to response to IL-6/sIL-6R. (*C*) Efficiency of clonal stem cell renewal mediated by chimeric receptors in response to G-CSF measured by formation of alkaline phosphatase positive colonies. Data are mean ± S.E.M. for duplicate assays on three independent clones normalized relative to response to IL-6/sIL-6R. (*D*) Electrophoretic mobility-shift assay of induced STAT3 DNA binding. Transfected clones were left untreated (lane *1*) or stimulated for 30 min with IL-6/sIL-6R (lane *2*) or with G-CSF at 30 ng/ml (lane *3*) or 3 ng/ml (lane *4*). Nuclear extracts were assayed for SIE binding. (*E*) Immunoblot of STAT3 and ERK phosphorylation induced by G-CSF stimulation of chimeric receptors. Transfected clones were left untreated (lane *1*) or were stimulated for 20 min with IL-6/sIL-6R (lane *2*) or with G-CSF (lane *3*). Immunoblots of cell lysates were probed sequentially with antibodies specific for the active phosphorylated forms of ERK and STAT3.

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The intact gp130 cytoplasmic domain mediated a clear induction of SIE DNA-binding activity (Fig. 2D). Mutation of individual docking sites had no appreciable effect. However, mutation of all four sites eliminated both the self-renewal signal and the induction of STAT3 DNA-binding activity. Radioligand binding established that cell surface expression was not limiting for any of the receptors (not shown). To confirm that other signaling pathways are not impaired by mutation of the STAT3 docking sites, we examined activation of the ERK cascade. ERK activation requires receptor phosphorylation on tyrosine 118 by JAK kinases and recruitment of SHP2 (Stahl et al. 1995; Fukada et al. 1996). Figure 2E shows that the basal level of constitutive ERK activity was significantly enhanced by stimulation of chimeric receptors in all transfectants tested. In particular, the two receptors, Y265/275F and Y126-275F, which gave reduced activation of STAT3 and cannot signal self-renewal, mediated normal and heightened levels of ERK activation, respectively. Therefore, there is no general compromise in the signaling capacity of these molecules.

Interestingly, this data also indicates that the STAT3 sites in gp130 may not be equivalent in vivo. Specifically, mutation of the two adjacent carboxy-terminal STAT3 binding sites (Y265 and Y275) abolished selfrenewal signaling, whereas mutation of the two-membrane proximal sites had little effect. This difference correlated with the lower induction of STAT3 DNA-binding activity and the specific reduction in STAT3 phosphorylation relative to ERK phosphorylation (Figs. 2D,E) (see Discussion). Self-renewal thus appears to require an appreciable level of STAT3 activation.

Inhibition of STAT3 activation blocks self-renewal and promotes differentiation

The above findings indicated that STAT3 may play a key role in ES cell signaling. To assess directly the requirement for STAT3 activation in ES cell self-renewal, we exploited a dominant interfering mutant form of STAT3, STAT3F. In this mutant (Minami et al. 1996), the tyrosine residue at amino acid position 705 is mutated to phenylalanine. Phosphorylation of Tyr705 is required for dimerization and nuclear translocation. When expressed at high levels, STAT3F has been shown to block the activation of endogenous STAT3 in various cell types, possibly by titrating out receptor docking sites (Fukada et al. 1996; Minami et al. 1996; Nakajima et al. 1996; Bonni et al. 1997; Ihara et al. 1997).

Using conventional transfection approaches, we were unable to recover ES cell transfectants showing stable high-level expression of STAT3F. In parallel experiments, however, transfection of the LIF-independent embryonal carcinoma cell line P19 yielded multiple expressing clones. This suggested that blockade of STAT3 activation in ES cells specifically resulted in cell death, growth arrest, or differentiation. An alternative transfection and expression strategy was therefore adopted to enable characterization of the consequences of STAT3F expression. The approach, termed supertransfection, relies on expression of polyoma virus large T protein by the recipient ES cells and its interaction with a polyoma origin of replication present in the transfected DNA. This results in efficient episomal propagation of incoming plasmid (Gassmann et al. 1995). We have developed this system for efficent cDNA expression in ES cells (H. Niwa, I. Chambers, L. Forrester, M. Gassmann, and A.G. Smith, in prep.). The process yields at least 100-fold more stable transfectants than conventional transfection protocols. A second important advantage of episomal supertransfection is that the unpredictable effects of chromosomal integration are avoided, with the result that the level of expression is both stable and relatively uniform (H. Niwa, I. Chambers, L. Forrester, M. Gassmann, and A.G. Smith, in prep.).

The STAT3F mutant cDNA was introduced into the supertransfection vector pHPCAG. The wild-type STAT3 coding sequence was also introduced, in both sense and antisense orientations. The three constructs were electroporated into MG1.19 cells that harbor a large T expression plasmid and can be supertransfected with constructs containing the polyoma origin (Gassmann et al. 1995). Supertransfectants were isolated by selection in hygromycin B for 8 days in the presence of LIF. Colonies were fixed, stained with Leishman's reagent, counted, and scored for the presence of stem cells and differentiated cells. More than 95% of colonies obtained following supertransfection with control or wild-type STAT3 vector were stem cell colonies (Fig. 3A). A modest increase in the proportion of differentiated colonies was obtained with the antisense construct. The STAT3F vector, however, yielded predominantly differentiated colonies. A decrease in total number of colonies was also observed after supertransfection with STAT3F. This may reflect an early onset of differentiation that would produce very small clones that would not be scored. Alternatively, very high levels of STAT3F expression may also be toxic, though this has not been reported in other cell types. Morphologically, the differentiated STAT3F colonies closely resembled the differentiated colonies generated on culture of ES cells in the absence of LIF (Fig. 3C). Various other cDNAs have been expressed in ES cells using this system, with little or no effect on formation of stem cell colonies (data not shown). This suggested that the effect on differentiation was specifically attributable to expression of STAT3F.

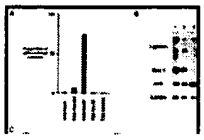
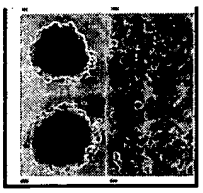


Figure 3. Induction of differentiation by expression of STAT3F in MG1.19 ES cells. (*A*) Proportion of differentiated colonies in LIF-supplemented medium resulting from supertransfection of STAT3, antisense STAT3, and STAT3F expression vectors. Colonies were fixed and stained with Leishman's reagent after 8 days of selection, and the numbers of stem cell colonies and differentiated colonies were scored. (*B*) Marker gene expression in STAT3F supertransfectants. Expression of marker genes in pools



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of MG1.19 cells supertransfected with STAT3 (lane *1*), STAT3 antisense (lane *2*), and STAT3F (lane *3*) expression vectors. Total RNA was prepared after 8 days of selection in LIF-supplemented medium, and 5-μg aliquots were analyzed by filter hybridization with β-globin, Rex-1, H19, and G3PDH probes. The β-globin probe detects all transgene mRNA species generated from pHPCAG, including an alternatively spliced product from the antisense contruct. (*C*) Photomicrographs of representative colonies 8 days after supertransfection with (*i*) STAT3, (*ii*) STAT3F, and (*iii*) empty expression vectors and selection in the presence of LIF, or (*iv*) induction of differentiation by culture in the absence of LIF for 8 days.

The differentiation induced by expression of STAT3F was examined further by expression analysis of the marker genes *rex1* and *H19*. Rex-1 mRNA, which is specifically expressed in undifferentiated stem cells, was down-regulated in STAT3F supertransfectants. In contrast, H19 RNA, which is found at low levels in stem cells but is up-regulated during differentiation, was increased (Fig. 3B). A similar pattern of gene regulation is observed during differentiation of ES cells induced by withdrawal of LIF. These data confirm that the morphological differentiation triggered by STAT3F is accompanied by reprogramming of gene expression.

STAT3F was also expressed from the mouse phosphoglycerate kinase (*pgk-1*) promoter in the episomal vector pHPPGK. This vector gives at least 10-fold lower expression than pHPCAG (H. Niwa, I. Chambers, L. Forrester, M. Gassmann, and A.G. Smith, in prep.). In this case, there was no significant effect on either colony number or differentiation status of MG1.19 supertransfectants. A relatively high level of expression of the dominant interfering mutant therefore appears necessary to block self-renewal.

Effect of STAT3F on self-renewal is suppressed by coexpression of STAT3

To test whether the induction of differentiation by expression of STAT3F was due to an inhibition of endogenous STAT3 activity, we attempted to rescue the stem cell phenotype by coexpression of wild-type STAT3 and also of STAT1 and STAT4. A STAT3F expression vector carrying a blasticidin resistance marker was cosupertransfected into MG1.19 cells with episomal constructs for expression of wild-type STATs and hygromycin resistance. Cosupertransfectants were isolated in medium containing both 20 μg/ml blasticidin S and 80 μg/ml of hygromycin B. The numbers of stem cell and differentiated colonies were scored after 8 days. As shown in Figure 4, only coexpression of wild-type STAT3 restored self-renewal in the presence of STAT3F. Transfection with STAT1 or STAT4 constructs alone had no effect on self-renewal in the absence of STAT3F (not shown) and did not alter differentiation induced by STAT3F. In the case of supertransfection with the CAG promoter STAT1 construct, the total number of colonies (stem plus differentiated) recovered was reduced, but the relative proportion of stem cell colonies versus differentiated cells was unaltered. This occurred in both the presence and absence of coexpression of STAT3F and suggests that high-level expression of STAT1 may be toxic to ES cells. By using the mouse PGK-1 promoter to drive lower levels of expression (H. Niwa, I. Chambers, L. Forrester, M. Gassmann, and A.G. Smith, in prep.), comparable numbers of colonies were recovered on transfection with the STAT1 as with the other constructs. In this case, again only the STAT3 construct showed any restoration of stem cell colonies, although to a lower degree than with the high-expression CAG vector (not shown). These data indicate that STAT3 has a specific function in ES cells that cannot be compensated by STAT1 or STAT4 (see Discussion).

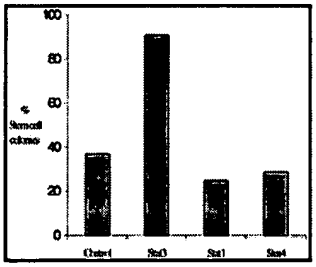

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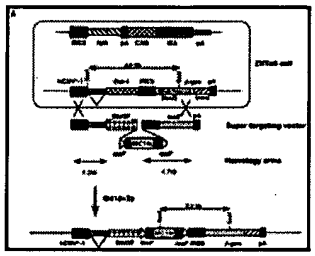
Figure 4. Cosupertransfection of STAT3F with wild-type STAT expression vectors. Proportions of undifferentiated stem cell colonies generated after cosupertransfection of MG1.19 ES cells with 10 μg of pBPCAGGS-STAT3F plus 10 μg of pHPCAG vector containing stuffer (control), STAT3, STAT1, or STAT4 inserts. After 8 days of selection with 80 μg/ml of hygromycin B plus 20 μg/ml of blasticidin S, colonies were fixed and stained with Leishman's reagent.

Generation of an inducible STAT3F transgene integration in ES cells

The effect of STAT3F expression on endogenous STAT3 activity could not be monitored directly in undifferentiated ES cells because ES cells expressing appreciable STAT3F constitutively could not be propagated. This required the generation of an inducible transgene. The tetracycline-regulatable system (tet-off) developed by Bujard and colleagues (Gossen and Bujard 1992) has been shown to confer inducibility on transgene expression in several cell types in culture and in the intact animal. However, it has proven problematic to establish this two-component system in ES cells. This is probably due to a combination of the relatively toxic effects of the tet repressor-

VP16 fusion (tTA) and the tendency of ES cells to suppress expression of integrated transgenes (silencing). We have isolated previously an ES cell line, ZHTc6, that maintains stable production of effective but nontoxic levels of tTA from a gene trap integration (H. Niwa and A. Smith, in prep.). This cell line also contains a tetracycline-responsive hCMV*-1 transgene integrated at a favorable expression site. Expression of such transgenes is usually deregulated and/or mosaic in ES cells because of the sensitivity of the hCMV*-1 promoter to site of integration effects and silencing. However, transgene expression in line ZHTc6 is completely repressed in the presence of tetracycline but is activated in all cells on withdrawal of tetracycline as revealed by β-galactosidase reporter expression (H. Niwa and A. Smith, in prep.). Because of the low efficiency of establishing de novo transgene integrations with such favorable characteristics, we adopted a transgene substitution approach to generate an inducible STAT3F transgene.

A targeting vector was designed for introduction of the STAT3F sequences into the hCMV*-1 locus by homologous recombination, using 5' and 3' sequences from the original transgenic construct as homology arms (Fig. 5A). In the presence of tetracycline, ZHTc6 cells are sensitive to G418 because the hCMV*-1 promoter is repressed. Advantage was taken of this by including a constitutive MC1 enhancer/promoter in the supertargeting vector to drive selectable marker expression. The absence of the *neo* sequence, however, requires that a legitimate recombination event with the resident transgene occur to confer G418 resistance. This powerful selection facilitated the isolation of targeted clones in which the STAT3F sequence was faithfully integrated 3' to the hCMV*-1 promoter (Fig. 5B). In the continued presence of tetracycline, the targeted cells were maintained readily as undifferentiated stem cell colonies in the presence of LIF. Three clones, Gs1, Gs2, and Gs3, were then analyzed further.



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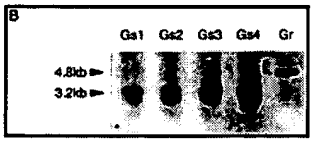

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Figure 5. Generation of an inducible STAT3F transgene integration by supertargeting. (*A*) Schematic of supertargeting strategy for introduction of STAT3F into a tetracycline-regulatable expression site. ZHTc6 ES cells contain a tetracycline-regulated transgene comprising the hCMV*-1 promoter (Gossen and Bujard 1992), β-globin second intron, Oct-4 open reading frame (Okazawa et al. 1991), and IRESβgeopA selection marker (Mountford et al. 1994). Homologous recombination can be used to replace the Oct-4 sequence (supertargeting). Use of a truncated selection marker in the targeting vector facilitates the isolation of homologous recombinants. ZHTc6 cells were electroporated with the STAT3F-SuperKO vector and selected in G418 in the presence of tetracycline. G418-resistant clones were duplicated and screened for sensitivity against gancyclovir to enrich further for homologous recombinants. The option of excising the *loxP*-flanked MC1tk cassette by transient expression of Cre recombinase was not pursued. (*B*) Diagnosis of the supertargeting event in Gs ES cells. Gancyclovir-sensitive (Gs; lanes *1-4*) and -resistant (Gr; lane *5*) clones were analyzed by Southern hybridization. A 3.2-kb *Sac*I fragment was detected with a probe from the 5' end of *lacZ* in the Gs samples, indicative of the correct replacement of the *Oct-4* cDNA sequence with STAT3F sequence. The Gr clone retained the 4.8-kb fragment diagnostic for the original *Oct-4* transgene integration in ZHTc6 cells.

Induced expression of STAT3F blocks ES cell self-renewal and causes differentiation

Withdrawal of tetracycline from Gs1, Gs2, or Gs3 cells resulted in the induction of differentiation in all three clones (Fig. 6A-C). Importantly, the efficiency of colony formation was not significantly different in the presence or absence of tetracycline, indicating that there is no toxic effect of STAT3F induction. The induced cultures differentiated over a 3- to 4-day time period, paralleling the behavior of parental ES cells on removal of LIF (Smith 1991). The differentiation response was confirmed by Northern hybridization analysis of Rex-1 and H19 transcripts (data not shown).

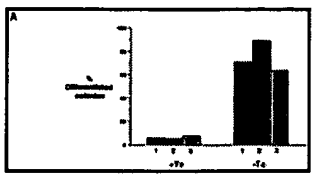

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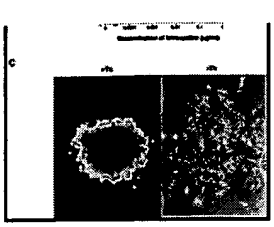
Figure 6. Induced expression of STAT3F causes ES cell differentiation and inhibits STAT3 activation. (*A*) Differentiation of Gs ES cells induced by withdrawal of tetracycline. Gs ES cells grown up in the presence of tetracycline were plated at clonal density (500 cells/60-mm dish) in LIF-supplemented medium in the presence or absence of tetracycline (1 μg/ml). After 6 days, colonies were fixed and stained with Leishman's reagent. The histogram records the proportions of differentiated colonies for three independent clones, Gs1 (solid bars), Gs2 (hatched bars), and Gs3 (shaded bars). (*B*) Dose response curve of Gs2 cell differentiation. Gs2 ES cells were cultured as above in the presence of the indicated concentrations of tetracycline, then fixed, stained, and scored. (*C*) Photomicrographs of uninduced and induced Gs2 ES cells. Representative colonies of Gs2 cells cultured for 6 days in LIF-supplemented medium in the presence (+Tc) or absence (−Tc) of tetracycline (1 μg/ml) and then fixed and stained with Leishman's reagent. (*D*) Mobility retardation assay of STAT3 DNA-binding activity in noninduced and induced Gs2 cells. Gs2 ES cells were cultured for 72 hr in the presence or absence of tetracycline. IL-6/sIL-6R was withdrawn for the final 24 hr, then restored for the indicated times. Nuclear



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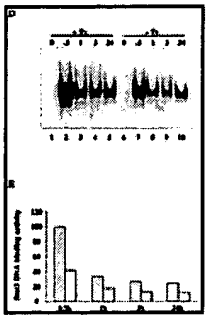

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extracts were prepared and assayed as described for SIE DNA-binding activity. (*E*) Quantitation of STAT3 SIE binding by PhosphorImager. (Shaded bars) +Tc; (open bars) −Tc.

Mobility retardation analysis was used to investigate directly STAT3 activation in STAT3F-expressing ES cells. The data in Figure 6D show that the level of STAT3 DNA-binding activity induced by gp130 stimulation was significantly lower in the presence of STAT3F. Quantitative PhosphorImager analysis confirmed a reduction of 50% or greater in the gel shift signal at all time points (Fig. 6E). The presence of residual STAT3 activity is consistent with the notion that a threshold level of active STAT3 is required to sustain self-renewal.

These findings confirm that expression of STAT3F in ES cells reduces gp130-mediated activation of STAT3, thereby blocking self-renewal and promoting differentiation.

Discussion

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The primary cytoplasmic signal transduction event emanating from a ligand-activated LIF-R/gp130 complex in ES cells as in other cell types is considered to be transphosphorylation and activation of receptor-associated Janus kinases (JAKs) (Davis et al. 1993; Narazaki et al. 1994). The JAKs then phosphorylate tyrosine residues in the receptors, creating docking sites for SH2 domain-containing proteins, notably including the STAT factors STAT1 and STAT3 (Lutticken et al. 1994; Stahl et al. 1995). STAT proteins are themselves targets for phosphorylation by JAKs, which leads to their dimerization and translocation to the nucleus. Other signal transducing molecules can also be activated downstream of gp130, including insulin receptor substrate-1 (IRS-1), phosphoinositide-3 kinases (PI-3 kinase), nonreceptor tyrosine kinases such as Hck and Btk, the tyrosine phosphatase SHP2, and the mitogen-activated protein kinases ERK1 and ERK2 (Boulton et al. 1994; Ernst et al. 1994; Yin and Yang 1994; Argetsinger et al. 1995; Matsuda et al. 1995a,b). This modular signaling system has been assumed to underlie the diverse and pleiotropic effects of IL-6 and LIF-related cytokines in different cell types. A key issue therefore is to resolve the relative contribution of different signaling pathways in any given responsive cell type. A critical role has been ascribed to SHP2-mediated activation of the MAP kinase cascade in proliferation of BAF-BO3 cells (Fukada et al. 1996) and suppression of apoptosis in cardiomyocytes (Sheng et al. 1997). In contrast, the differentiation responses of myeloid M1 cells (Minami et al. 1996; Nakajima et al. 1996) and primary neural precursors (Bonni et al. 1997) are effected via activation of STAT3. Previous studies in ES cells have suggested that JAK-STAT signaling, ERK activation, and the nonreceptor tyrosine kinase Hck could all be involved in LIF signaling (Ernst et al. 1994, 1996; Narazaki et al. 1994; Hocke et al. 1995; Boeuf et al. 1997).

We initially investigated the ability of chimeric receptor constructs to signal ES cell self-renewal by isolation of stably expressing transfectants. The observation that G-CSF-R can support ES cell propagation drew attention to signaling features conserved between G-CSF-R and gp130, notably the induction of STAT3 DNA-binding activity. Combined substitutions of the tyrosine residues in the STAT3 binding sites of gp130 cytoplasmic domain were associated with different levels of STAT3 activation and indicated that a self-renewal signal is associated with a threshold of STAT3 activity. Moreover, the four STAT3 sites do not appear to act in either a redundant or simple cumulative manner. Both self-renewal signaling and induction of STAT3 DNA-binding activity were maintained on pairwise

mutation of the two-membrane proximal STAT3 docking sites (Y126 and Y173) but not on mutation of the carboxy-terminal pair (Y265 and Y275) (see Fig. 2). This observation is somewhat unexpected as it has been shown previously that the isolated phosphopeptide sequences have equivalent STAT3 binding properties (Stahl et al. 1995) and that a truncated receptor with a single-membrane proximal STAT3 site (Y126) can efficiently induce STAT3-mediated differentiation of M1 cells (Yamanaka et al. 1996). It is important to note, however, that in the truncated receptor, sequences that mediate receptor internalization (Dittrich et al. 1996) have also been deleted with unpredictable consequences for signaling properties. Our findings indicate that in the normal context of the full-length receptor, the four STAT3 docking sites are not equivalent. The explanation for the reduced activity of the membrane proximal pair of sites is unclear though one possibility is that availability of Y126 may be influenced by interaction of SHP2 with Y118 (note enhanced ERK activation from Y126-275F chimera in Fig. 2E).

The finding that mutation of the STAT3 binding sites in the cytoplasmic domain of gp130 abolished the self-renewal signal prompted a direct investigation of the role of this transcription factor. New strategies were required to express the dominant interfering mutant STAT3F in ES cells. The methods we have deployed in this study enhance the experimental versatility and tractability of ES cells and establish new avenues for the characterization in vitro of gene functions involved in stem cell propagation, commitment, or differentiation. Because of the >100-fold increase in stable transfection efficiency and the relative homogeneity of expression (H. Niwa, I. Chambers, L. Forrester, M. Gassmann, and A.G. Smith, in prep.), episomal supertransfection provides a methodology for the screening and analysis of cDNAs whose expression is not compatible with ES cell self-renewal. The first demonstration of effective operation of the tetracycline regulation system in ES cells provides a complementary inducible expression approach. These two methods should find broad application in functional screening and in the genetic manipulation of lineage commitment and differentiation processes in ES cells.

Both constitutive expression of STAT3F following episomal supertransfection and induced expression from the regulatable chromosomal site inhibited self-renewal and resulted in differentiation. The episomal approach also allowed the specificity of the requirement for STAT3 to be established by coexpression of various STAT family members with STAT3F. The finding that STAT3 can restore self-renewal indicates that this factor serves a specific and nonredundant function in ES cell self-renewal in response to LIF. The evidence that STAT1 cannot compensate for STAT3 is noteworthy because STAT1 can be activated in response to LIF in ES cells, though to a much lesser extent than STAT3 (Starr et al. 1997). STAT1 may play little or no role in ES cell propagation. Induction of STAT1 DNA-binding activity was not evidently associated with self-renewal signaling from the various chimeric receptors used in this study (Figs. 1B and 2D). Furthermore, ES cells in which both alleles of the *stat1* gene have been inactivated are phenotypically normal (Durbin et al. 1996).

A role for STAT3 in ES cell signaling has recently also been suggested by Boeuf et al. (1997) who reported the isolation of ES cell clones expressing STAT3F constitutively. These cells apparently showed an increased tendency to differentiate after 1 month or more in culture. The basis of this phenomenon is unclear because absence or blockade of LIF signaling results in complete differentiation within a few days (Smith et al. 1988; Williams et al. 1988; C. Dani, I. Chambers, S. Johnstone, M. Robertson, B. Ebrahimi-Chahardahcherik, M. Saito, T. Taga, M. Li, T. Burdon, J. Nichols, and A.G. Smith, in prep.). We were unable to establish conventional transfectants expressing significant levels of STAT3F. However, our data on both episomal and induced expression demonstrate that STAT3F rapidly and efficiently blocks ES cell self-renewal and triggers differentiation.

Our results establish that STAT3 activation is essential for LIF-R/gp130-mediated ES cell self-renewal. STAT3 activity is regulated by phosphorylation on both tyrosine and serine (Wen et al. 1995), and a constitutively active mutant has not been described. An isoform of STAT3, STAT3β, generated by alternative splicing, is reported to show sustained activation properties (Schaefer et al. 1995). ES cells supertransfected with a STAT3β vector remained LIF dependent (data not shown), however, indicating that this isoform does not substitute for activated STAT3 in ES cells. This may be because STAT3β appears to function by formation of heterodimers with c-Jun (Schaefer et al. 1995), and it is anticipated that the STAT3β/c-Jun complex regulates a distinct spectrum of target genes compared with the STAT3 homodimer. It is noteworthy, however, that expression of v-*src* in ES cells renders them LIF independent (Boulter et al. 1991). v-Src has been shown to associate with and cause constitutive activation of STAT3 (Cao et al. 1996).

The p42/p44 MAP kinase pathway (ERK1 and ERK2) has been reported to be activated by LIF in ES cells as in other cell types (Ernst et al. 1996; Boeuf et al. 1997). The Ras-ERK cascade is coupled to gp130 via the adaptor molecule SHP2 (Fukada et al. 1996; Yamanaka et al. 1996). SHP2 interacts with activated gp130 at phosphorylated tyrosine residue 118 (Stahl et al. 1995). Significantly, mutation of this residue does not inhibit self-renewal signaling in ES cells (T. Burdon, I. Chambers, C. Stracey, J. Nichols, and A.G. Smith, in prep.). Furthermore, the MEK inhibitor PD098059 (Dudley et al. 1995) that specifically blocks activation of the ERK kinases does not inhibit stem cell colony formation in response to LIF (T. Burdon, I. Chambers, C. Stracey, J. Nichols, and A.G. Smith, in prep.). Thus, although contributions of other pathways are not precluded, STAT3 appears to play a central role in ES cell self-renewal. The underlying importance of STAT3 is further attested to by the finding that homozygous disruption of the *Stat3* gene in mice is associated with early embryonic lethality (Takeda et al. 1997).

It is striking that the role of STAT3 in propagation of the undifferentiated pluripotential phenotype of ES cells contrasts with previously characterized functions as an effector of somatic cell differentiation. Dominant interfering mutants of STAT3 have been shown to block

macrophage differentiation of myeloid M1 cells induced by IL-6 or LIF (Minami et al. 1996; Nakajima et al. 1996) or by GCSF (Shimozaki et al. 1997). STAT3 activation has similarly been shown to mediate IL-6- or LIF-induced astrocytic differentiation of primary cortical neuroepithelial cells (Bonni et al. 1997). Recently it has also been shown that STAT3 is activated by hepatocyte growth factor and mediates epithelial tubulogenesis (Boccaccio et al. 1998). STAT3 thus has distinct effects in different cell types. A common theme, however, may be the regulation of genes that determine cell identity. The diverse effects of the LIF/IL-6 family of cytokines on cellular differentiation and gene expression appear to reflect cell-type specific effects of active STAT3. In the context of stem cell propagation, the key issue now is to identify transcriptional targets of STAT3 in ES cells and to illuminate the relationship between STAT3 and the essential ES cell-specific transcription factor Oct-4.

Materials and methods

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Cell culture and transfection

ES cells were maintained in the absence of feeder cells in Glasgow modification of Eagle medium (GMEM) supplemented with fetal calf serum, 2-mercaptoethanol, and LIF (Smith 1991). CGR8 (Mountford et al. 1994) and MG1.19 (Gassmann et al. 1995) ES cells have been described elsewhere. DO27 ES cells have had both copies of the *lif* gene inactivated by homologous recombination and the IRESβgeo selection marker/reporter inserted into the *oct4* gene as described (C. Dani, I. Chambers, S. Johnstone, M. Robertson, B. Ebrahimi-Chahardahcherik, M. Saito, T. Taga, M. Li, T. Burdon, J. Nichols, and A.G. Smith, in prep.). LRKOh34 ES cells have targeted disruptions in both copies of the *lifr* gene (M. Li, I. Chambers, J. Nichols, and A.G. Smith, in prep.) and are maintained in medium in which LIF is substituted with IL-6 (50 ng/ml) and soluble IL-6 receptor (5% CHO-5E7 conditioned medium; Yasukawa et al. 1990). For conventional transfection with pPCAGIZ vectors, 1×10^7 cells were electroporated with 100 μg of linearized plasmid DNA at 800 V and 3 μF in a 0.4-cm cuvette using a Bio-Rad gene pulser and then selected in the presence of zeocin (Invitrogen). For transfection of episomal vectors (supertransfection), 5×10^6 MG1.19 cells were electroporated with 20 μg of supercoiled plasmid DNA at 200 V and 960 μF and then cultured in the presence of either 80 μg/ml hygromycin B (Boehringer Mannheim) or 4-20 μg/ml blasticidin S (Waken Seiyaku), or both hygromycin plus blasticidin for cosupertransfection.

Generation of tetracycline regulatable transgenes in ES cells

ZHTc6 ES cells were derived from CGR8 ES cells (Mountford et al. 1994) and will be described in detail elsewhere (H. Niwa and A.G. Smith, in prep.). They carry a targeted integration of IRES*zeo* in one *Oct3/4* allele. They also carry a gene trap integration of an IRES*hph:*CAGtTA construct that confers stable expression of the tetracycline-responsive tTA transactivator and a randomly integrated hCMV*-1-Oct4-IRESβgeopA transgene. These cells were routinely maintained in the presence of 10 μg/ml zeocin and 1 μg/ml tetracycline-HCl (Sigma).

The hCMV*-1-Oct-4-IRESβgeopA transgene is comprised of the tetracycline-inducible promoter hCMV*-1 derived from pUHD10-3 (Gossen and Bujard 1992), rabbit β-globin second intron, full-length Oct-4 cDNA, and IRESβgeopA unit (Mountford et al. 1994). pSuperKO (see Fig. 5A) contains the hCMV*-1 and rabbit globin sequences as the 5' homology arm and the IRES*lacZ* cassette as 3' arm. Intervening are a stuffer sequence with *Xho*I and *Sfi*I cloning sites and a *loxP*-flanked MC1*tk* cassette (Mansour et al. 1988). The STAT3F cDNA was introduced as a *Sal*I fragment between the *Xho*I sites. For gene targeting, 2×10^7 cells were electroporated with 100 μg linearized SuperKO-STAT3F plasmid DNA at 800 V and 3 μF and then selected in the presence of 200 μg/ml G418 (GIBCO BRL) and 1 μg/ml tetracycline-HCl. Targeted clones were maintained in the continuous presence of tetracycline-HCl.

Plasmid construction

DNA manipulations were performed by standard procedures (Sambrook et al. 1989). Full details of plasmid constructions are available on request. The full-length mouse G-CSF-R cDNA (pJ17) was provided by Shigekazu Nagata (Fukunaga et al. 1990), and the G-CSF-R/LIF-R chimeric receptor construct (Baumann et al. 1994b) was provided by Steve Ziegler. G-CSF-R/gp130 chimeric receptor constructs were generated by fusing the coding sequence for the extracellular domain of human G-CSF-R (Baumann et al. 1994b) to an *Eco*RI fragment encoding the transmembrane domain and the entire cytoplasmic region of mouse gp130 cDNA (Hibi et al. 1990). Phenylalanine substitutions were introduced into the intracellular domain of gp130 by PCR overlap mutagenesis (Higuchi et al. 1988). PCR products were substituted into the G-CSF-R/gp130 chimera and sequenced. Episomal expression vectors pHPCAG, pBPCAG, and pHPPGK are described elsewhere (H. Niwa, I. Chambers, L. Forrester, M. Gassmann, and A.G. Smith, in prep.). The expression vector pPCAGIZ, which can be used as both an episomal and an integrated expression vector, was constructed by ligation of the encephalomyocarditis virus IRES (pCITE-1, Novagen) with the *Streptoalloteichus* bleomycin resistant gene (*Sh ble:zeo*) from pZeoSV (Invitrogen) and introduction into pPCAG (H. Niwa, I. Chambers, L. Forrester, M. Gassmann, and A.G. Smith, in prep.). cDNAs are inserted into a *Xho*I site 5' to the IRES. The requirement for continuous relatively high-level expression of the *zeo* gene to confer antibiotic resistance allows direct selection for integrations into favorable expression sites. Consequently, using this vector, ES cell transfectants can readily be isolated that sustain stable transgene expression (H. Niwa, T. Burdon, I. Chambers, and A.G. Smith, unpubl.).

RNA and DNA hybridization analyses

Total RNA (Chomczynski and Sacchi 1987) was separated on a 0.66 M formaldehyde, 0.8% agarose gel and blotted onto nylon membranes (Hybond N, Amersham). Hybridization was performed with β-globin third exon, Rex-1, H19, and GAPDH cDNA probes labeled by random hexamer primed DNA synthesis in the presence of [α-^{32}P]dCTP (3000 Ci/mmole).

For identification of targeted ES cell clones, genomic DNA was digested with *Sac*I, separated on a 0.7% agarose gel, and analyzed by nonradioactive filter hybridization (Gene Image, Amersham) with an *Eco*RI-*Sac*I fragment of the *lacZ* gene.

G-CSF-R binding assay

ES cells (1×10^6) were seeded in wells of a 24-well plate and grown for 24 hr. The cells were then cooled to 4°C and growth medium was replaced with 0.25 ml of ice-cold binding buffer (GMEM, 25 mM HEPES at pH 7.2, 0.2% BSA) containing 0.212 nM ^{125}I-labeled G-CSF-R (Amersham) in the presence or absence of a 1000-fold molar excess of cold G-CSF-R. Binding reactions were incubated for 3 hr at 4°C and terminated by washing the cells three times with ice-cold binding buffer. Cells were then solubilized in 0.5% NP-40, and an aliquot was counted in a gamma counter. All treatments were performed in duplicate. No specific binding was detected to untransfected cells, and nondisplaceable binding was consistent between clones.

Self-renewal assays

To measure self-renewal of ES cells at cloning density, cells were plated at 1000 cells per well (~100 cells/cm^2) in 6-well dishes and cultured for 6 days. Cells were either grown in the absence of cytokines, in 100 U/ml recombinant LIF (Smith 1991), in 100 ng/ml IL-6 plus soluble IL-6R, or in 30 ng/ml G-CSF-R, as appropriate. On day 6, colonies were fixed and stained with Leishman's reagent (Smith 1991) or for alkaline phosphatase activity (Sigma leukocyte alkaline phosphatase kit) (Bernstine et al. 1973). Numbers of stem cell and differentiated colonies were scored by microscopic examination, in some cases with computer-assisted image analysis. All assays were performed in duplicate or triplicate.

Stem cell-specific expression of β-galactosidase from the *oct4* locus in D027 cells was quantified by ONPG assay on triplicate samples. Cells were plated at 5000 per well in 24-well dishes and cultured for 6 days in the presence or absence of cytokine as above. On day 6, cells were washed once with PBS and lysed in 0.4 ml of 0.25 M Tris (pH 7.5), 5 mM DTT, and 0.5% NP-40. Lysate (40 μl) was mixed with 100 μl of ONPG buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 1.2 mM ONPG) in a microtiter plate and incubated at 37°C for 2-4 hr, and the absorbance was read at 420 nm.

Preparation of nuclear extracts and band-shift assays

One day after plating (1×10^6 cells per 60-mm dish), ES cells were washed with PBS and refed with medium lacking cytokines. The next day, cells were stimulated with IL-6 (100 ng/ml plus soluble receptor) or G-CSF-R (30 ng/ml) for 30 min, washed with ice-cold PBS, scraped off the plates, and collected by centrifugation. Nuclear extracts were prepared by the method described (Gobert et al. 1996) except that protease inhibitors (aprotinin, pepstatin, and leupeptin) were omitted from the cell lysis buffer. Protein concentrations of nuclear extracts were determined using a Bradford assay (Bio-Rad). Aliquots (2 μg) of nuclear extract were incubated with 0.25 ng of ^{32}P-labeled double-stranded SIEm67 oligonucleotide probe (Sadowski et al. 1993) in binding buffer (20 mM HEPES at pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.05% NP-40, 10% glycerol, 2 μg/ml of poly[d(I-C)], and 1 mg/ml BSA) for 20 min at room temperature. Binding reactions were resolved by electrophoresis on a prerun 5% polyacrylamide gel in 0.25× TBE for 3 hr. Gels were fixed in 10% acetic acid, dried under vacuum, and subjected to autoradiography or quantitated on a Bio-Rad PhosphorImager.

Immunoblotting

One day after plating (1×10^6 cells per 60-mm dish), ES cells were refed with medium containing 1% FCS and lacking cytokines. Following overnight incubation, cells were transferred to serum-free medium for 4 hr prior to stimulation with IL-6 (100 ng/ml plus soluble receptor) or G-CSF-R (30 ng/ml) for 20 min. Cells were then washed once with ice-cold PBS and lysed on ice in 100 μl SDS sample buffer. Ten- microliter aliquots of the lysates were fractionated on a 10% SDS-polyacrylamide gel and electroblotted onto nitrocellulose. After overnight treatment in blocking buffer (25 mM Tris-HCl at pH 7.4, 2.7 mM KCl, 140 mM NaCl, 0.1% Tween 20, 5% nonfat dried milk), membranes were probed sequentially with the phospho-specific anti-ERK and anti-STAT3 antibodies according to the directions provided by the supplier (New England Biolabs). Blots were incubated with HRP-coupled anti-rabbit IgG and developed using ECL reagents (Amersham). Membranes were stripped between probings by incubation at 50°C for 30 min in 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-mercaptoethanol.

Acknowledgments

We thank Alexander Medvinsky and John Bishop for comments on the manuscript. Craig Stracey is thanked for help with β-galactosidase assays, and Melany Jackson for assistance with image analysis. DO27 and LRKOh34 cells were generated by Christian Dani and Meng Li, respectively. We are grateful to Shizuo Akira and Daniel Nathans for the STAT3F and STAT3β constructs, respectively, James Darnell for STAT1 and STAT4 cDNAs, K. Akagi and Hermann Bujard for the tTA and hCMV*-1 constructs, and

Tetsuya Taga for gp130 cDNA and recombinant IL-6 and sIL-6R. Recombinant G-CSF-R was a generous gift of Chugai Corporation. Photographic reproductions were by Graham Brown and colleagues. This work was supported by the Biotechnology and Biological Sciences Research Council of the United Kingdom, the Human Frontiers Science Program Organisation, and Stem Cell Sciences Pty.

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Footnotes

Received December 1, 1997; revised version accepted April 23, 1998.

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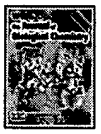

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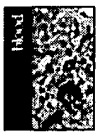

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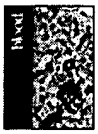

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(51) International Patent Classification 5 :
C12N 15/90, A01K 67/027, C12N 15/00, 5/10, 15/12, 15/19, 15/15

A1

(11) International Publication Number: WO 94/24301

(43) International Publication Date: 27 October 1994 (27.10.94)

(21) International Application Number: PCT/GB94/00849

(22) International Filing Date: 21 April 1994 (21.04.94)

(30) Priority Data:

9308271.7	21 April 1993 (21.04.93)	GB
9313323.9	28 June 1993 (28.06.93)	GB
9401011.3	20 January 1994 (20.01.94)	GB

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(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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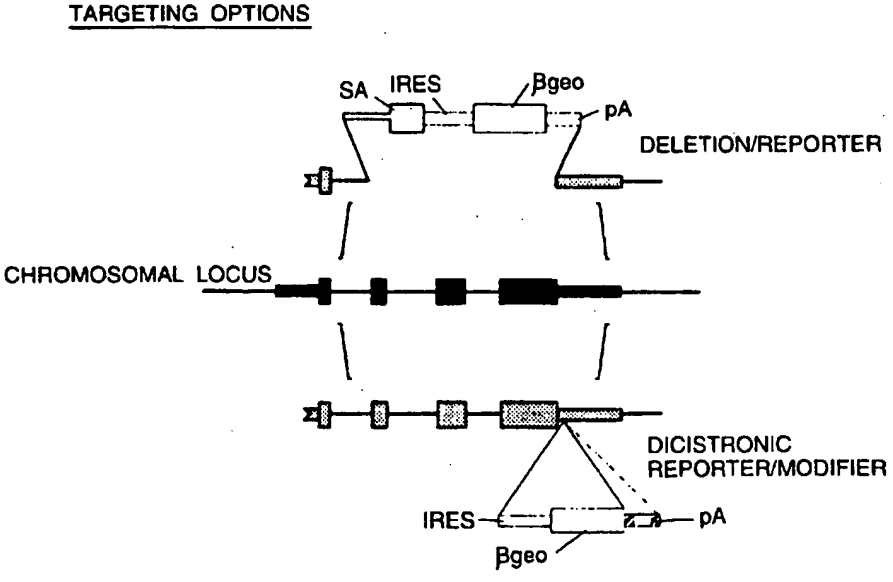
(54) Title: EXPRESSION OF HETEROLOGOUS GENES ACCORDING TO A TARGETED EXPRESSION PROFILE



(57) Abstract

A DNA construct is described containing an expression unit of an internal ribosome binding site (IRES) coupled to a heterologous gene sequence; this expression unit is bounded at 5' and 3' ends by DNA sequences that enable homologous recombination or integration of the construct with the DNA of a targeted host to obtain expression of the heterologous gene in the host.

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EXPRESSION OF HETEROLOGOUS GENES ACCORDING TO A TARGETED EXPRESSION PROFILE.

This invention relates to DNA constructs for inserting heterologous gene sequences into a host genome so as to obtain expression of the heterologous gene, to methods of inserting heterologous gene sequences into a host genome and to organisms carrying modified host genomes.

In one particular aspect this invention relates to constructs for inserting a heterologous gene into an endogenous gene in a host genome so that the heterologous gene is expressed in place of or in addition to the endogenous gene. In a second particular aspect this invention relates to methods for functionally integrating a heterologous gene sequence (transgene) into a specified gene of a host genome so as intimately to couple transgene expression with the endogenous transcriptional and post-transcriptional regulatory elements, to constructs for use in said methods, and to genetically modified cells and transgenic animals generated with such constructs and their descendants.

Genetic engineering involves the fusion of different gene sequences. In many cases this is performed with the intention of expressing a heterologous gene sequence in a fashion which is identical to or in part reflects the expression pattern of another gene. To achieve the desired expression level, distribution and/or timing or the sequence being expressed, regulatory sequences of the gene being copied are fused with the sequences of the gene which is to be expressed to generate an expression construct. However, in many applications involving higher eukaryotic cells, such as the selection of particular stem cells or the production of heterologous proteins from transgenic animals, it is extremely difficult to generate an expression construct whose pattern and level of expression adequately mimics those of the gene being copied.

It is known to introduce heterologous genes into mammalian cells including stem cells, transgenic animals and *in vitro* maintained cell lines. However, despite specific design, existing expression constructs, when integrated into the host genome, rarely provide the desired level and distribution (both spatial and temporal) of gene expression. Expression constructs are known that attempt to mimic the expression profile of an endogenous gene by incorporating known regulatory elements of the endogenous gene. However, success with these constructs is low partly because functional detail of the endogenous gene structure including the location and identity of such elements and the contribution each component makes in regulating gene expression, for the most part, remains unknown. Other problems are associated with randomly integrating expression constructs including positional effects of the site of integration and random mutation of endogenous gene expression.

Furthermore, to position and define regulatory elements in endogenous genes, often at some distance from the transcribed region of the gene, often demands much painstaking work. The distal positioning of these elements is also often important to their function and may be difficult to reproduce in transgenic expression constructs.

Further still, having identified and engineered the endogenous regulatory elements into heterologous gene expression constructs, there is little assurance that any particular transgenic expression construct will function correctly once introduced at random into the genome.

Early attempts to produce heterologous proteins in transgenic animals principally focused on the use of transgene constructs comprising promoter regions derived from one gene fused to cDNA coding sequences from another gene. For the most part the fusion constructs function poorly, if at all, and the level of expression obtained is far lower than that of

the endogenous gene.

This is in contrast with intact genes, such as the ovine whey protein betalactoglobulin (BLG). High-level expression of the encoded protein is obtained in transgenic mice harbouring a full-length BLG gene complete with all introns and adequate lengths of 5' and 3' untranscribed regions (Simons et al., Nature 328,530-532,1987).

Attempts were made by various groups to harness the efficient expression of such genomic transgenes to drive the expression of heterologous coding sequences in transgenic animals. Tandem gene constructs are not normally expressed in mammalian systems because only the first (upstream) coding sequence is translated. For this reason most workers were obliged to fuse, into the 5' untranslated region (5'UTR) of the genomic gene, a cDNA coding for the heterologous protein of interest.

Tomasetto et al. (Mol. Endocrinol. 3, 1579-1584,1989) fused a pS2 cDNA into the 5'UTR of the whey acidic protein (WAP) gene. Although some expression was observed, the production level was extremely low. Similarly, Simons et al. (Bio/Technology 6, 179-183,1988) produced constructs in which cDNA's encoding human factor IX or alpha-1 antitrypsin were introduced into the 5'UTR of ovine BLG. Both in transgenic mice and transgenic sheep these constructs failed to function properly, with only low levels of expression being obtained (Clark et al., Bio/Technology 7, 487-492,1989).

Although some reports indicate that the simple insertion of intron sequences into expression constructs can augment expression (eg. Brinster et al., Proc. Natl. Acad. Sci. 85, 836-840,1988) the level of expression remains low compared with that of the endogenous gene, suggesting that intron sequences per se are not sufficient to permit high-level gene expression in a transgenic context. This is confirmed by the results of Whitelaw et al. (Transgenic Res. 1, 3-13,1991) who

deleted the introns from the BLG gene and then added back a single intron. The intron-less gene was poorly active and the presence of a single intron was not sufficient to restore the transcriptional efficiency of the BLG gene in transgenic mice.

It has been argued that the overall gene structure, including the relative positions of introns and exons, is critically important for transgene function. This contention is wholly supported by the finding that the 5' end of the BLG gene, when fused to a genomic copy of the human alpha-1 antitrypsin gene, leads to consistent high-level expression in transgenic animals (Archibald et al., Proc. Natl. Acad. Sci. USA 87, 5178-5182,1990).

In practice, however, it is often difficult to apply this genomic fusion technology. Many genes of particular interest are extremely large (eg. the human factor VIII gene is over 100 kilobases in length) and the generation of fusion constructs, and their introduction into transgenic mammals (including livestock) is extremely difficult.

An alternative to engineering expression constructs (by coupling regulatory elements of one or several gene/s with the heterologous gene sequence to be expressed) *in vitro*, is to utilise the "gene trap" approach. Regulatory elements controlling expression of gene trap expression constructs, are provided by inserting the heterologous sequence which is to be expressed, into a gene in the host's genome. Sequences of the gene to be expressed are thereby intimately coupled with the regulatory elements of the endogenous gene.

By far the great majority of gene trap type vectors are used for random integration or trapping of host genes, with the disadvantage that there is no control over the site of integration or the generation of endogenous gene/transgene fusion products. One gene trap vector, pGT4.5 is known from

Genes & Development 6:903-918 by Cold Spring Harbour Laboratory Press, 1992.

A major limitation in the design and functional utilisation of all "gene trap" and "genomic transgene" expression constructs known in the prior art, is the mechanism of transgene translation initiation. Translation of most mRNAs is initiated by a scanning mechanism in which a ribosome complex (termed 43S) binds at the 5' end of capped mRNA and moves along the mRNA until a suitably placed AUG initiation codon is detected. Subsequently a second ribosome subunit (termed 60S) joins the complex and protein synthesis begins.

In 1988, Pettetier and Sonenberg (Nature, 334:320-325) showed that some picornavirus mRNAs are translated by an unusual mechanism of "internal ribosome binding" and that these particular mRNAs contained specific sequences internal to the mRNA that enabled a ribosome to bind and initiate translation. The sequences were termed "Internal Ribosome Entry Site" (IRES). Picornaviruses infect human cells so this work indicated that eukaryotic ribosomes recognised the IRES and could initiate translation internally, and other than via a cap-dependent mechanism.

Ghattas *et al* (Molecular & Cellular Biology, Vol. 11 No. 12, Dec. 1991, pp5848-5859) describe the use of an internal ribosome entry site in obtaining co-expression of two genes from a recombinant provirus in cultured cells and in chicken embryos.

However, there currently exists no efficient procedure by which a heterologous gene sequence (transgene) to be expressed in eukaryotic cells, in particular mammalian stem cells, transgenic animals or cultured cells, can be inserted into the genome of a host cell so as to obtain expression of that heterologous gene in a desired pattern, one example of a desired pattern being intimately to couple expression of the

heterologous gene with regulatory elements controlling expression of a targeted endogenous gene.

It is an object of the invention to provide a DNA construct and methods for its use that enable improved efficiency of heterologous gene expression in a host cell. To provide the heterologous gene expression at a desired level is another object. A further object is to provide expression with a desired temporal and/or spatial profile during the life of a host cell or population of cells or transgenic organism.

By "heterologous gene expression" is meant both (1) expression in a host of a gene that was previously not expressed in that host, and (2) expression in a host of a gene according to a particular expression profile, the gene being previously expressed in the host but not according to the particular expression profile.

Accordingly, in a first aspect the invention provides a DNA construct for inserting a heterologous gene sequence into a host genome, the construct comprising the following sequence:

5' X-A-P-B-Q-C-Y 3'

in which	
X and Y	are, separately, DNA sequences substantially homologous with a host gene locus,
P	is an internal ribosome entry site (IRES),
Q	is the heterologous gene sequence, and
A, B and C	are optional linker sequences.

X and Y should be of sufficient length and homology with host sequences to enable homologous recombination to take place between the DNA construct of the invention and the corresponding host genome DNA. It is preferable that X and Y are each at least 1000 base pairs. However, it will be

appreciated that, in general, while effective homologous recombination is in some instances achieved with X and Y having rather short sequences, efficiency will be increased as the length of the sequences increases.
X and Y are preferably at least 95% more preferably at least 98%, and most preferably substantially 100% homologous with the host.

In embodiments of the invention, X and Y (i) together constitute a DNA sequence substantially homologous with a single continuous host DNA sequence or (ii) are substantially homologous with two separate sequences from the same endogenous host gene locus and in the same respective orientation as in the endogenous locus. In a preferred embodiment, the DNA construct is part of a vector capable of transforming a host cell by inserting the DNA construct into the host cell DNA.

P, the IRES, is 5' to the open reading frame of the heterologous gene sequence Q. Where B is absent, the IRES is immediately 5' to the open reading frame of the heterologous gene.

The linker regions A, B and C are additional DNA sequences optionally present in the DNA construct. The linker regions may be inserted into the construct or may arise as a result of the recombinant DNA techniques used in making the construct. In an embodiment of the invention linker region A includes or consists of a splice acceptor. The size and nature of linker B in particular is important in providing an optimal linkage between the IRES and the heterologous gene (Cell, Vol.68, pp119-131, January 1992).

To select for successful transformants expressing the heterologous gene it is convenient to include a selectable marker, for example an antibiotic resistance gene or a hypoxanthine ribosyl transferase gene, in the heterologous

gene. Including a selectable marker enhances the probability of selecting transfected cells with the desired transgene integration as expression of the selectable marker is dependent upon functional integration into an active gene. Transgene integrations in non-transcribed regions of the genome are therefore readily eliminated.

When a construct according to the invention is used to transform a host genome, homologous recombination with the host DNA results in insertion of the construct into a host gene. Transcription of the heterologous gene is then under control of the regulatory elements associated with the host gene. Translation of the heterologous gene coding sequence is then enabled by the presence of the IRES 5' to the open reading frame of the heterologous gene. This results in regulated expression of the heterologous gene with considerably greater efficiency than under hitherto known and used techniques for obtaining heterologous gene expression.

In use, a heterologous gene and an endogenous gene with a particular pattern and/or level of expression in a host cell are selected. A DNA construct is made having X and Y substantially homologous to parts of the endogenous gene or to flanking regions of the endogenous gene. The DNA construct will then target insertion of the heterologous gene plus IRES into (or in place of) that endogenous gene so that heterologous gene transcription is directed by the host regulatory elements for that endogenous gene. Translation of mature heterologous gene product is enabled by the IRES included in the DNA construct and newly inserted along with the heterologous gene.

The utilisation of IRES-mediated translation initiation in gene trap type targeting vectors according to the invention provides a considerable advantage over previously described gene traps and gene trap targeting vectors in that functional integration of the transgene into the desired endogenous gene

transcribed region does not produce a fusion protein and need not necessarily disrupt endogenous gene expression.

Octamer binding transcription factor 4 is a member of the POU family of transcription factors (reviewed by Schöler, 1991). Oct4 transcription is activated between the 4- and 8-cell stage in the developing mouse embryo and it is highly expressed in the expanding blastocyst and then in the pluripotent cells of the inner cell mass. Transcription is down-regulated as the primitive ectoderm differentiates to form mesoderm (Schöler et al., 1990) and by 8.5 d.p.c. (days post coitum) is restricted to migrating primordial germ cells. High level Oct4 gene expression is also observed in pluripotent embryo carcinoma and embryonic stem cell lines, and is down-regulated when these cells are induced to differentiate (Schöler et al., 1989; Okamoto et al., 1990).

The Oct4 gene was selected as a suitable example of the use of the constructs of the invention because of the known moderate to high levels of Oct4 mRNA. Results show that despite a down-regulation in transcription from the targeted Oct4 allele, consistent with the removal of a possible enhancer sequence in the second intron, the Oct4 gene can be targeted at very high efficiency using the methods and constructs of the invention.

In one embodiment of the invention integration of a transgenic construct incorporating an IRES element and an open reading frame into a position 3' to the stop codon and 5' of the polyadenylation signal generates a functional dicistronic mRNA capable of encoding both the endogenous gene product and the product of the transgenic open reading frame. In another embodiment transgene integration 5' to or in place of the endogenous gene reading frame provides an opportunity to "knock-out" (or otherwise modify) the endogenous gene product.

Analyses of eukaryotic genes in many laboratories have shown

that in general the coding sequences of DNA, the regions that will ultimately be translated into amino acid sequences, are not continuous but are interrupted by 'silent' DNA. Even for genes with no protein product, such as tRNA genes of yeast in Drosophila, the primary RNA transcript contains internal regions that are excised during maturation, the final tRNA or mRNA being a spliced product. The regions which will be lost from the mature messenger are termed "introns" (for intragenic regions) and alternate with regions which will be expressed, termed "exons". Transgenes may be fuctionally inserted into exons, or in a further aspect of the invention, incorporate a splice acceptor sequence 5' to the IRES element to enable fuctional integration into an intron. Functional transgene integration is therefore not restricted by the intron/exon arrangement or reading frame of the endogenous gene. This is another aspect in which the design and construction of transgenic constructs of the invention is simpler than that of hitherto known constructs.

The IRES containing vectors of the invention enable gene targetting with increased efficiency. The invention permits a heterologous gene coding sequence to be inserted into the 3' untranslated region of a gene (3'UTR), therefore conserving the relative positions of all the upstream introns and exons, and leading to high-level expression. The requirement for a genomic copy of the heterologous gene is avoided, and successful expression can be obtained by inserting a cDNA copy downstream of the IRES in the 3'UTR. Because cDNAs are very much shorter that the corresponding genomic copy, the assembly of constructs and the generation of transgenic mammals is considerably facilitated.

In a preferred embodiment the heterologous gene includes at its 3' (downstream) end a polyadenylation signal . An advantage of this embodiment is that the polyadenylation signal results in efficient truncation and processing of the

transcript at the end of the heterologous gene.

In particularly preferred embodiments the DNA construct also includes a truncation/ cleavage/ transcription termination sequence 5'(upstream) of the homologous region X. The function of the 5' sequence is to prevent mRNA read-thrrough; suitable sequences include a poly A signal, such as the SV40 polyadenylation signal, and the Upstream Mouse Sequence (UMS) (Heard et al., 1987). The 5' sequence can further include a splice acceptor. It is known that DNA constructs can integrate at random into the host genome, i.e. that they do not always insert by homologous recombination with the targeted endogenous gene. Random integration into any active gene can result in heterologous gene expression; this makes it difficult to recognize correct insertion events, which is a disadvantage. The particularly preferred embodiments overcome this problem because where random integration occurs the transcription termination or truncation or cleavage sequence also integrates, blocking transcription. It is advantageously found that where homologous recombination occurs with the targeted endogenous gene, the transcription blocking sequence does not integrate, so transcription of the heterologous gene is possible.

In these particularly preferred embodiments of the invention are established methods effectively to eliminate expression after random gene trap integration events and thereby provide a gene trap type targeting strategy which enables selection specifically for the desired targeting event. This method is termed by the inventors *Positive Only Selection (POS)* and utilises transcript truncation/ cleavage sequences (e.g. polyadenylation sequences) or transcriptional termination sequences such as the UMS, to block expression of the transgene in the event of random integration into actively transcribed genes. Homologous recombination with the target gene functionally inserts the heterologous gene and, if present, a selectable marker, but

not the upstream transcriptional termination sequence, and therefore permits transcription of the heterologous gene and, where present, the selectable marker.

Thus "POS" embodiments of the invention extend the potential of the gene trap expression technology by providing methods of essentially eliminating expression of the transgene from sites of integration other than the desired target gene. The POS system has particular application in gene therapy where restricting transgene expression to the targeted locus would be of enormous value.

Using the DNA constructs of the first aspect it is possible to insert a heterologous gene into an endogenous host gene so that the start of the heterologous gene sequence is inserted substantially at the start of the endogenous target gene sequence. In such cases the IRES is optionally omitted, i.e. the DNA construct comprises:

5' T-D-X-A-Q-C-Y 3'

wherein T is a transcription terminator or truncator,
D is an optional linker sequence, and
X, Y, A, C and Q are as previously defined.

The constructs of the invention are also advantageous for addressing the problem of expressing in a target host cell or organism (which we designate for clarity as cell "T") a gene ("G") according to particular expression profile ("E") where endogenous genes with a suitable expression profile are not present or are not accessible. The solution is to identify a donor host cell ("D") that includes a gene ("H") with expression profile E and to create a construct according to the invention in which X and Y are of such length that they include the cell D elements that regulate expression of the endogenous gene in cell D according to profile E. The DNA construct thus includes (1) the cell D regulatory elements for

a targeted endogenous gene, the expression profile E of which is desired to be mimicked, (2) an IRES and (3) a heterologous gene sequence G. The DNA construct is allowed randomly to integrate into the cell T DNA.

Random integration of the construct into the cell T DNA generates a modified cell T expressing the heterologous gene according approximately to expression profile E of cell D. The result is expression of the gene in cell T with a similar pattern to that of H in cell D.

Following random integration of the DNA construct of the invention into cell T, the modified cell T is target for DNA constructs according to any embodiment of the invention operating via homologous recombination.

In a second aspect the invention provides a method of inserting a heterologous gene into a target endogenous gene in a host cell genome comprising transforming a host cell with a DNA construct according to the first aspect of the invention. Transformation can include introducing the DNA of the invention into a cell or preparation of cells by transfection, by injection ballistics, by plasmid or viral vector or by electroporation or by fusion.

In a third aspect the invention provides a method of expressing a heterologous gene in a host cell by making a DNA construct according to the first aspect of the invention comprising the heterologous gene, allowing the DNA construct to undergo homologous recombination with the host genome and growing a culture of host cells expressing the heterologous gene.

The invention thus provides a method of using promoterless transgenic constructs flanked by regions of gene homology,

such that homologous recombination between DNA of a transgenic construct and the target gene locus leads to functional insertion of the transgene into the chosen transcription unit. Transcription of the transgene is regulated by elements associated with the endogenous gene, and/or additional elements introduced to the site with the transgene. Translation of the transgenic reading frame or frames is mediated via cap-independent translation initiation through the incorporation of an internal ribosome entry site/s (IRES) immediately 5' to the open reading frame/s. This provides an exquisite level of transgene regulation and avoids many of the problems associated with the design and successful utilisation of previously described expression constructs for transgene expression.

In a fourth aspect the invention provides a method of expressing a heterologous gene in a host cell by making a promoterless DNA construct according to the invention, allowing it to undergo random integration with the host genome and growing a culture of cells expressing the heterologous gene.

In a fifth aspect the invention provides a method of experssing a heterologous gene in a host cell by engineering a functional expression construct prior to introducing the construct into the host genome. In an embodiment one such "genomic transgene" is engineered *in vitro* by inserting an IRES coupled to a heterologous gene which is to be expressed, into a large genomic sequence (for example a cosmid or an artificial chromosome encompassing the gene which is to be copied) which incorporates most if not all regulatory elements of the gene. In another embodiment, a genomic transgene is engineered *in vitro* by targeting IRES and heterologous gene which is to be expressed, into the endogenous host gene and subsequently isolating from the targeted cell line a large genomic fragment (for example, cosmid or artificial chromosome) which incorporates the IRES and sequence to be

expressed and most if not all of the regulatory elements associated with the targeted gene. Large genomic tansgenes then provide the desired transgene expression following random introduction into the host cell.

In a sixth aspect the invention provides a transgenic cell or transgenic organism or transgenic animal into the genome of which a heterologous gene has been inserted using a DNA construct according to the invention either by homologous recombination or by random integration. In a seventh aspect the invention provides descendants of the sixth aspect that have inherited the heterologous genes. The invention is applicable to heterologous gene expression in both eukaryotes and prokaryotes, though preferably eukaryotes and more preferably animal cells; and mammalian cells in particular.

Obviously the utility of the constructs and methods of the invention in selecting for the desired integration event is limited to introducing transgenic constructs which incorporate a selectable marker gene into endogenous genes which are expressed at sufficient levels in the cells being transfected. To introduce a non-selectable gene into an actively transcribed gene for expression independently of a selectable marker, the target locus would first be "marked" with a construct according to the invention expressing a selectable marker which can be both selected for (primary targeting) and selected against (secondary targeting). Once marked through a primary targeting event, transgene integrations into the "marked" gene could be selected for by the absence of the primary targeting gene selectable marker. This type of approach is particularly applicable where repetitive targeting of a particular gene is envisaged such as in the development of cell lines or transgenic animals for the over-expression of heterologous genes.

If the gene being targeted is not sufficiently expressed for primary gene trap "marking", promoter mediated expression of a

selectable marker may be similarly employed in standard non-gene trap type targeting vectors to mark the target gene.

In a particularly preferred embodiment of the invention vectors have been constructed which employ encephalomyocarditus virus (EMCV) IRES-mediated translation of a LacZ/bacterial neomycin resistance fusion gene (ßgeo, Freidrich and Soriano, 1991) for gene targeting in murine embryonic stem (ES) cells. Translation of the ßgeo fusion gene generates a bifunctional gene product which provides both reporter and selectable marker gene activity. Vectors were designed to target and subsequently report (a) normal Differentiation Inhibiting Activity/Leukaemia Inhibitory Activity (DIA/LIF) gene expression by non-disruptive insertion of the transgene 3' to the endogenous gene reading frame, and (b) altered DIA gene expression resulting from a defined modification at the DIA locus, an (c) altered ocamer-binding transcription factor 4 (Oct4) expression resulting from a defined modification at the locus.

DIA is a pleiotropic cytokine which suppresses differentiation of ES cells *in vitro* and has been implicated in a variety of developmental and physiological processes *in vivo*. The DIA gene was selected as a suitable example of the use of constructs of the invention because of the known low levels of DIA mRNA. Results show that despite low steady state DIA mRNA levels (<10 copies/cell) the DIA gene can be targeted at high efficiency.

These results suggest therefore, that the use of constructs according to the invention is applicable at least in ES cells to genes expressed even at low levels.

To investigate whether IRES-mediated translation efficiency is cell type dependent, we generated a random gene trap vector according to the invention which utilises the EMCV-IRES to initiate translation of the ßgeo fusion gene. Neomycin

resistant cell lines which display LacZ staining in a variety of differentiated cell types were selected for blastocyst injection and the subsequent generation of chimaeras. Chimaeras were bred to provide fully transgenic animals for analysis of LacZ expression profile. This analysis should provide valuable insight into the efficiency of IRES-mediated translation in other cell types.

There now follow descriptions of exemplary embodiments of the invention in which

Figs. 1-3 and 6 illustrate DNA constructs of the invention,
Figs. 4 and 5 show DNA constructs for use in making the constructs.
Figs. 7 and 8 show the IRES-βgeo Targeting Strategy:
Fig 7-Schematic representation of internal initiation of translation mediated through the IRES in a dicistronic transcript.
Fig 8-applications of the IRESβgeo cassette in gene targeting. Constructs can be designed either to delete all or part of a gene whilst incorporating the *lacZ* reporter, or to append the reporter with or without modification of the intact gene, and
Figs. 9-12 show DNA and mRNA Hybridisation Analyses of Targetted Clones:
Figure 9-DIA/LIF targeting. Genomic DNA digested with Hind III or Eco RI was hybridized with either an exon 1-specific 163bp Xho I-Eae I fragment from pDR100 or with a 700bp Pst I-Eco RI 3' genomic fragment respectively. Lane 1, CGR8 parental ES cells; lanes 2, 5 and 6, clones targetted with the non-truncating construct; lanes 3 and 4, clones targetted with the truncating construct.

Figure 10-Oct-4 targeting. Primary screen on genomic DNA prepared in agarose plugs by Eco RI digestion and hybridisation with a 5' 587bp Nco I fragment, and confirmatory hybridisation with a 600bp Hind III-Sau 3A 3' fragment following Cla I digestion of phenol/chloroform-extracted DNA.

Cla I reproducibly gave partial digestion of the introduced site, suggestive of variable methylation within the *lacZ* sequence. Lane 1, parental CGR8 ES cells; lane 2, non-targetted transfectant; lanes 3-7, targetted clones.

Figure 11 Detection of fusion transcripts in ES cell clones with targetted integrations at the DIA locus. In order to increase the level of DIA expression, ES cells were induced to differentiate by exposure to 10^{-6}M retinoic acid. Poly(A$^+$) enriched RNA was prepared after 4 days, applied to a formaldehyde gel and transferred to nylon membrane. The filter was hybridized with a 650bp DIA/LIF coding sequence probe and exposed for 21 days, then stripped and rehybridised with an 800bp *lacZ* fragment. Lane 1, RNA (1.5µg) from parental CGR8 cells; lane 2, RNA (3µg) from cells targetted with the non-truncating construct; lanes 3 and 4, RNA (3µg) from cells targetted with the truncating construct.

Figure 12-Detection of fusion transcript in Oct-4 targetted ES cells. Total RNA was prepared from undifferentiated ES cells. The Oct-4 probe was a 408bp Nco I-Pst I 5' cDNA fragment (292) which contains only 24bp of exon 2 and should therefore give equivalent hybridisation to wild-type and fusion transcripts.

Fig. 13 shows steps in the generation of a construct of the invention as described in Example 3.

EXAMPLE 1

DIA gene targeting constructs (Figures 1 and 2) were designed to integrate transgenes which express the ß-geo fusion gene product so as to provide gene expression under the control of the endogenous DIA gene locus. A third construct (Figure 3) was designed to demonstrate the advantages gained through transcriptional blockers which, when engineered into gene trap targeting constructs at a position 5' to the DNA targeting homology, greatly reduce if not eliminate expression from

randomly integrated transgenes.

ES Cell Culture and Manipulation

ES cells were routinely maintained as described (by Smith, A. G. (1991) *J. Tiss. Cult. Meth.* 13, 89-94) in the absence of feeders in medium supplemented with murine DIA/LIF. The germline competent cell line CGR8 was established from strain 129 embryos by published procedures (Nichols, J., Evans, E. P. & Smith, A. G. (1990) *Development* 110, 1341-1348). Aggregation chimaeras were produced between ES cells and outbred MF1 embryos by a modification of the method of Wood *et al.* (Wood, S. A., Pascoe, W. S., Schmidt, C., Kemler, R., Evans, M. J. & Allen, N. D. (1993) *Proc. Natl. Acad. Sci. USA* 90, 4582-4585) in which co-culture is performed in hanging drops. For germ-line transmission, chimaeras were produced by blastocyst injection. For isolation of homologous recombinants, 10^8 cells were electroporated with 150µg linearised plasmid at 0.8kV and 3µFd in a 0.4cm cuvette, then selected in the presence of 175µg/ml G418. Genomic DNA was prepared in agarose plugs (Brown, W. R. A. (1988) *EMBO J.* 7, 2377-2385) from 24-well plate cultures while duplicate plates were stored frozen (Ure, J., Fiering, S. & Smith, A. G. (1992) *Trends. Genet.* 8, 6). To assay DIA/LIF production, ES cells were induced to differentiate by incubation with 6mM 3-methoxybenzamide and conditioned media was harvested and assayed for the ability to inhibit ES cell differentiation as described. The assay was rendered specific for DIA/LIF by inclusion of a neutralising polyclonal antisera raised against murine DIA/LIF (AS, unpublished). Histochemical staining for β-galactosidase was carried out using X-gal (Beddington, R. S. P., Morgenstern, J., Land, H. & Hogan, A. (1989) *Development* 106, 37-46) and fluorescent staining was performed with DetectaGene Green (Molecular Probes) according to the manufacturer's instructions.

Plasmid Construction

DNA manipulations were carried out following standard procedures. The IRES is a 594bp sequence from the 5' untranslated region (UTR) of EMCV mRNA which has been modified by mutagenesis of the native initiation codon. Translation is initiated by an ATG which lies 9bp 3' of the normal start site and forms part of the Nco I cloning site.

Briefly, the IRESβgeo cassette was constructed by ligating a 5' fragment of the EMCV-IRES/*lacZ* fusion (Ghattas et al., 1991) to 3' *lacZ/neo*^{R} sequences of the *βgeo* gene fusion (Friedrich, G. & Soriano, P. (1991) *Genes Dev.* 5, 1513-1523). The pGTIRESβgeopA plasmid was then generated by 5' ligation of the *en-2* splice acceptor (Gossler, A., Joyner, A. L., Rossant, J. & Skarnes, W. C. (1989) *Science* 244, 463-465) and 3' ligation of SV40 polyadenylation sequences. Targeting constructs were prepared from genomic clones isolated from a strain 129 λ library. DIA/LIF targeting constructs were generated within a 7kb fragment extending from a Sac II site between the alternative first exons to a Hind III site 3' of the gene. The DIA-βgeo construction was prepared by insertion of the IRESβgeo cassette into the unique Xba I site. To generate the DIA-βgeopA construct, a 1.2kb Bam HI fragment containing 3' βgeo sequences and SV40 polyadenylation sequences was isolated from pGTIRESβgeopA and ligated into the Bam HI digested DIA-βgeo construct. This results in insertion of the 200bp SV40 sequences in place of a 400bp fragment of DIA/LIF 3' UTR. The Oct-4 targeting construct contained 1.6kb of 5' homology, extending from a Hind III site within the first exon to an Xho I site in the first intron, and 4.3kb of 3' homology extending from the Nar I site 3' of the polyadenylation sequence to a Hind III site.

In detail, to generate the DIA targeting constructs a preliminary vector coupling the EMCV-IRES to the βgeo fusion gene was engineered. This was generated by ligating a 1.2 kb Bam HI fragment encompassing the bacterial Neomycin resistance gene (*neo*) and the SV40 polyadenylation signal into the Bam HI

site of the Bluescript II KS(-) cloning vector (Stratagene) to generate *vector "1"*. Independently, a 1.4 kb Bgl II/Cla I fragment encompassing the EMCV-IRES and 5' LacZ sequences was isolated from pLZIN (Ghattas *et al.*, 1991) and ligated into pGT1.8βgeo to generate the vector designated *pGT1.8IRESβgeo* (Figure 4). A 4.9 kb Xba I fragment encompassing the entire IRESβgeo fusion gene was isolated from *pGT1.8IRESβgeo* and ligated into Xba I digested *vector "1"* to generate *IRES-βgeo (for targeting)* (Figure 5).

To generate the *DIA-IRESβgeo* targeting vector (Figure 1) the 4.9 kb Xba I IRES-βgeo fragment *from IRES-βgeo(for targeting)* (Figure 5) was ligated into a unique Xba I site overlapping the translational stop codon of the murine DIA gene. The murine DIA gene fragment used in the design of the DIA gene trap targeting vectors spanned from a Sac II site immediately 3' to the alternate first exon (encoding the "D" transcript) to a Hind III site approximately 7 kb 3' of this site.

The second DIA gene targeting vector designated *DIA IRESβgeo pA* was generated by inserting the SV40 polyadenylation sequence immediately 3' to the IRESβgeo transgene. This was accomplished by inserting a Bam HI neo/pA fragment from *IRES-βgeo(for targeting)* into Bam HI digested *7kb DIA IRESβgeo*. The resultant construct was identical to the *7kb DIA IRESβgeo* targeting construct except for the inclusion of the SV40 polyadenylation signal in place of approximately 400 bp of DIA gene 3' UTR sequence.

The *"POS" DIA IRESβgeo* targeting vector was generated by inserting a 1400 bp Nco I/Pst I pSVTKNeob fragment, incorporating the rabbit β-globin gene splice acceptor and exon sequences and the SV40 polyadenylation signal, into the Sac II site at the 5' extremity of the DIA gene DNA homology (Figure 3).

The Oct4-neo construct (Oct4-tgtvec) designed for targeted

integration into the Oct4 gene is shown in Figure 6. This construct incorporates 1.6 kb of 5' Oct4 gene sequence, 4.3 kb of 3' Oct4 gene sequence a *lacZ-neomycin* fusion gene (ßgeo, encoding a bifunctional protein, Freidrich and Soriano, 1991) into the first intron of the Oct4 mRNA. Splicing from the splice donor sequence of the first exon-intron boundary to the integrated IRES-ßgeo sequence is facilitated by the inclusion a murine *engrailed-2* splice acceptor sequence (Skarnes *et al.*, 1992) immediately 5' to the IRES-ßgeo sequence. Translation of the ßgeo cistron of the Oct4-ßgeo fusion transcript is facilitated by the inclusion of the EMCV-IRES immediately 5' to the ßgeo coding sequence.

ES cell transfection and colony selection:

Mouse 129 ES cells (line CGR-8) were prepared and maintained in the presence DIA as described by Smith (1991). Plasmid DNA for transfection was linearised by Sal I digest, ethanol precipitated and resuspended at 10-14 mg/ml in PBS. Following 10 hours culture in fresh medium, near confluent ES cells were dispersed by trypsinisation, washed sequentially in culture medium and PBS, and resuspended at $1.4x10^8$/ml in PBS for immediate transfection. Routinely, 0.7 ml of cell suspension was mixed with 0.1 ml DNA containing solution and electroporated at 0.8 kV and 3.0 µFD using a Biorad Gene Pulser and 0.4 cm cuvettes. Transfections were plated on gelatinised tissue culture dishes at $5-8x10^4/cm^2$ in growth medium for 16 hours prior to the addition of selection medium containing 200 µg/ml (active) G418 (Sigma). Single colonies were picked 8-10 days post transfection and transferred in duplicate into 24 well tissue culture plates for further expansion in growth medium containing 200 µg/ml G418.

Once confluent, one series of cells were frozen for storage while the remainder were analyzed by Southern analysis and/or lacZ staining.

Further characterisation of the DIA gene-targeted cell lines:

Selected cell lines were assayed for lacZ staining patterns following ES cell growth and differentiation in DIA-supplemented medium, or following retinoic acid induced differentiation in non-DIA-supplemented medium.

Production of chimaeras from the DIA gene-targeted cell lines:

Selected cell lines were cultured in the absence of G418 for 7 days prior to embryo injection as previously described (Nichols *et al.*, 1990). Briefly, blastocysts for injection were collected 4 d.p.c. from C57/Bl6 donors, injected with 10-20 cells and allowed to re-expand in culture prior to transfer to the uteri of pseudopregnant recipients. Chimaeras were identified by the presence of patches of sandy coat colour on the C57/BL6 background. Male chimaeras may be test bred for transmission of the transgenes. Transgenic mice may be analyzed for lacZ staining.

DNA and RNA Hybridisation Analyses

Filter hybridisations were performed on nylon membranes according to standard procedures using random-primed ^{32}P-labelled probes. Homologous recombinants were characterised with probes from both 5' and 3' flanking sequences. Whole mount *in situ* hybridisation with digoxigenin-labelled Oct-4 antisense RNA (Schöler, H., Dressler, G. R., Balling, R., Rohdewold, H. & Gruss, P. (1990) *EMBO J.* 9, 2185-2195) was performed essentially as described (Wilkinson, D. G. (1992) in *situ hybridization: a practical approach*, ed. Wilkinson, D. G. (IRL Press, Oxford), pp. 75-83).

The steady state level of DIA/LIF mRNA in ES cells is fewer than 10 copies per cell; this provided a stern test of the general utility of IRES targeting vectors of the invention. Targeting vectors were constructed by introduction of the

IRES-*βgeo* module at the Xba I site which overlaps the stop codon (Fig. 9). The entire coding sequence was thus left intact and intron sequences were unaltered. Two constructs were built, DIA-βgeo and DIA-βgeopA, which differed by inclusion of the SV40 polyadenylation signal 3' of the *βgeo* sequence. The fusion transcript generated following homologous recombination with the former construct utilises the endogenous 3' UTR and polyadenylation signal of the DIA/LIF gene, whereas the DIA-βgeopA construct gives rise to a truncated transcript lacking these sequences.

In contrast to DIA/LIF, both mRNA and protein for the octamer-binding transcription factor Oct-4 (also known as Oct-3), are relatively abundant in ES cells. Oct-4 is also found in oocytes, pluripotential early embryo cells and primordial germ cells. The association of Oct-4 with pluripotency is strengthened by its rapid down-regulation during differentiation. An IRES-*βgeo* vector was designed both to generate a null allele and to introduce an expression marker into the Oct-4 locus (Fig 8). The latter could facilitate the detection of hitherto unidentified sites of Oct-4 expression. The POU-specific domain and the homeodomain coding sequences in exons 2 to 5 were deleted and replaced by the IRES-*βgeo*pA module (Fig. 11). Since the 5' arm of homology ended within the first intron, the *en-2* splice acceptor sequence was included 5' to the IRES in order to facilitate productive splicing from exon 1 after homologous recombination.

Following electroporation and selection in G418, individual clones were analyzed by Southern hybridisation with both 5' and 3' flanking probes to detect replacement targeting events (Fig. 9-12) and with internal probes to monitor for multiple integrations. The frequencies of homologous recombination obtained with the constructs of the invention are presented in Table 1.

Correct replacement events were observed with all vectors. A

particularly high frequency was reproducibly obtained at the Oct-4 locus. This may reflect the high expression level of this gene in ES cells in addition to the contributions of isogenic DNA and the enrichment afforded by a promoterless construct. Targeting of DIA/LIF with the poly(A) addition vector was also efficient. The isolation of correctly targetted clones at the DIA/LIF locus establishes that IRES-mediated translation is applicable to genes expressed at very low levels in ES cells.

Northern analyses of several targetted clones confirmed that all contained fusion transcripts of the predicted sizes (Fig.s 11,12) which hybridised to both *lac Z* and DIA/LIF or Oct-4 probes respectively. The transcript generated by non-truncating insertion of IRES-*βgeo* into the DIA/LIF gene in clone D70 was detected in similar, although slightly lower, amounts to the normal transcript. This indicates that the IRES-*βgeo* sequence itself does not have any profound influence on either transcription or message turnover. The truncated fusion species produced upon integration of IRES-*βgeo*pA was 5-fold more abundant by phosphorimage scanning than the normal message. The increased level of fusion transcript in these cells was reflected in the production of biologically active DIA/LIF protein; 3-6-fold more DIA/LIF was present in conditioned medium prepared from differentiated cultures of cells with targetted truncations than from the parental cells or cells targetted with the non-truncating construct. Thus the fusion transcript is a functional dicistronic mRNA and the targeting event has modified the activity of the targeted gene. The Oct-4 fusion transcript on the other hand was 10-20-fold less abundant than wild-type Oct-4 mRNA. This could be attributable to inefficient utilisation of the *en-2* splice acceptor, but might also arise from deletion of either stabilising elements within the mRNA or an enhancer within the gene.

The *in vitro* studies illustrate the potential of the constructs and methods of the invention for obtaining targeted heterologous gene expression.

EXAMPLE 2

To address the issue of tissue-specificity of IRES function we made a series of random IRES gene traps according to the invention by electroporation of pGTIRESβgeopA into ES cells. Several clones which exhibited widespread expression of β-galactosidase in differentiated cell types *in vitro* were used to produce aggregation chimaeras. At 7.5 and 8.5 days of development, β-galactosidase could be detected in all tissues colonised by the ES cells, that is throughout the embryo and in the amnion and visceral yolk sac. These gene traps have been transmitted through the germ line, confirming that the presence of the IRES is compatible with functional gametogenesis, and preliminary analyses on the heterozygotes indicate that the IRES is functional in a wide variety of embryonic and adult tissues. Aggregation chimaeras have also been produced with the Oct-4 targetted cells. The staining pattern of such embryos at 7.5 days shows that the tissue-specific distribution of Oct-4 mRNA is accurately reflected by the β-galactosidase expression pattern.

Example 3

Application of the invention to the efficient expression of heterologous molecules by insertion of an IRES and a cDNA into the 3' untranslated region of a genomic clone of a tissue-specific gene and the generation of transgenic animals by microinjection into fertilised eggs.

In the following example a cDNA (eg. human alpha-1 antitrypsin) is inserted, downstream of an IRES (eg. from EMCV), into the 3' untranslated region of a genomic gene that functions efficently and in a tissue-specific manner in

transgenic animals (eg. the ovine beta-lactoglobulin gene, BLG).

The IRES from encephalomyocarditis virus (EMCV) is available as a 600 bp EcoRI-NcoI fragment, where the NcoI site (CCATGG) defines the start site of translation; it also contains a HindIII site introduced some nucleotides upstream of the NcoI site, changing the spacing between the IRES and the ATG (Ghattas et al., Mol. Cell. Biol. 11, 5848-5859, 1991). First, the upstream EcoRI site is converted, by linker insertion (sequence GAATTGATATCAATT) to an EcoRV site. Two versions of the IRES are employed, one (IRES-1) in which the heterologous coding sequence is introduced at the NcoI site, a second in which site-directed mutagenesis is used to position the ATG within the NcoI site 20 nucleotides downstream of box A (TTTCC, Pilipenko et al., Cell 68, 119-131, 1992), removing the HindIII site (the DNA sequence in this region now reading TTTCCTTTGAAAAACACGATAACCATGG) (Fig. 13, A). The modified IRES is termed IRES-2. IRES-1 and IRES-2 are both used, as EcoRI-NcoI fragments, for the following experiments.

The ovine BLG gene is present on a large SaII-SaII fragment (or, alternatively as a slightly smaller SaII-XbaI fragment) (Simons et al., Nature 328, 530-532, 1987; Ali and Clark, J. Mol. Biol. 199, 415-426, 1988; Harris et al., Nucl. Acids Res. 16, 10379, 1988) cloned into pPolyIII-I (Lathe et al., Gene 57, 193-201, 1987). Both fragments express at high level in lactating mammary gland when introduced into transgenic animals (Simons et al., Nature 328,530-532, 1987).

Immediately downstream of the translation stop codon in the last exon lies a unique AatII site (GACGT/C). This site is converted, by insertion of a linker, to an EcoRV site (final sequence GACGTGATATCACGTC) (Fig. 13, D). Although this construction is based on the use of the entire SaII-SaII fragment, the SaII-XbaI fragment may also be used with appropriate minor modifications to the procedure.

The reporter gene used in this experiment is human alpha-1 antitrypsin cDNA though the procedure can be repeated with any other cDNA. The cDNA is engineered, by localised mutagenesis, such that an NcoI site overlaps the initiating ATG (this may lead to a single base change in the second codon, so changing the nature of the amino acid encoded at this position. Because in most cases this amino acid does not contribute to the mature protein because it is at the beginning of the signal sequence this has no adverse consequences for expression, secretion or activity of the mature protein). Similarly, an EcoRV site is engineered at the 3' terminus of the cDNA such that the 3' untranslated region is removed (sequence at the 3' terminus of the cDNA reading TAAGATATC, where the stop codon TAA could be TAA, TAG or TGA) (Fig. 13, B). The NcoI-EcoRV fragment (obtained, where necessary, by partial digestion in cases where internal sites are present) is used in the following experiments.

Next, pPolyIII-I (Lathe et al., Gene 57, 193-201, 1987) is modified such that a synthetic BamHI-SaII-PstI polylinker is inserted between the BamHI and PstI sites (sequence of polylinker - GGATCCGCGTCGACCACTGCAG; restriction sites are underlined) (Fig. 13, C). The SaII-SaII fragment encompassing the modified (EcoRV site at the place of the AatII site) genomic ovine BLG gene is cloned into the SaII site. The IRES and the modified cDNA are excised as EcoRV-NcoI and NcoI-EcoRV fragments respectively, ligated together, and the fusion product EcoRV-NcoI-EcoRV inserted into the EcoRV site within 3' untranslated region of the BLG gene (Fig. 13, E).

The hybrid molecule, BLG-IRES-AAT-BLG, is exercised from the plasmid with SfiI or another appropriate enzyme and microinjected into fertilised eggs of mouse or sheep. Transgenic animals harbouring this construct, for the most part, are observed to express high levels of AAT in their milk. Constructs of the invention could also be used to obtain expression of other proteins of biomedical importance.

* * * * * * * * *

The experiments reported here establish that the use of IRES-targeting according to the invention is a powerful means of expressing a desired gene in a host genome. Moreover, the IRES configuration used in these studies was not optimal for translation of the 3' cistron. It has been found that the precise location of the ATG relative to the 3' end of the IRES has a major effect on translational efficiency. It appears that production of βgeo could be increased several-fold over that achieved in the present study. This should increase the ability to isolate recombinants in poorly expressed genes and enhance the sensitivity of the *lac Z* reporter.

The IRES-targetting strategy of the invention is a powerful means of reporting and modifying mammalian gene expression. Furthermore, it is apparent that non-disruptive integration of an IRES-linked marker into a 3' UTR provides a convenient means for introducing subtle mutations into a gene. Moreover, the IRES strategy is not limited to modification of endogeneous genes and the introduction of reporters, but is also applicable to the controlled expression of transgenes. The desired specificity and levels of transgene expression could be ensured by the use of IRES-mediated translation either in genomic constructs for pronuclear injection or following homologous integration into an appropriate locus. The latter could be achieved by the construction of polycistronic vectors containing two IRES elements. Alternatively, sequential rounds of homologous replacement or targetting followed by recombinational deletion of the selectable marker could be employed to introduce an IRES expression cassette with mimimal disruption into any genes which are not expressed in ES cells. In general therefore, the flexibility and utility of IRES-mediated translation seem likely to find widespread application in transgenic research.

Table 1 Frequency of Isolation of Homologous Recombinants with IRES vectors.

Construct	Cell Line	Colonies Screened	Number Positive	Percent Positive
Oct4-βgeo	CGR8	51	44	86%
"	E14TG2a	10	7	70%
"	D1C2	30	21	70%
DIA-βgeopA	CGR8	79	21	26%
DIA-βgeo	CGR8	109	3	2.7%
"POS" DIA-Bgeo	CGR8	20	20	100%

CLAIMS

1. A DNA construct for inserting a heterologous gene sequence into a host genome comprising the sequence:

5' X-A-P-B-Q-C-Y 3'

in which

X and Y	are substantially homologous with respective portions of the host genome
P	is an internal ribosome entry site (IRES),
Q	is the heterologous gene sequence,
A, B and C	are, separately, optional linker sequences.

2. A DNA construct according to Claim 1 in which X and Y are of sufficient length to undergo homologous recombination with the host genome so as to insert the A-P-B-Q-C sequence into the host genome.

3. A DNA construct according to Claim 2 in which X and Y are each at least 1000 base pairs in length.

4. A DNA construct according to Claim 1, 2 or 3 in which X and Y are both homologous with a part of an endogenous host gene.

5. A DNA construct according to Claim 4 in which X and Y comprise the host elements regulating expression of the endogenous gene.

6. A DNA construct according to any preceding claim in which all of the linker sequences A, B, and C are absent.

7. A DNA construct according to any preceding claim additionally comprising a polyadenylation signal at the 3'

(downstream) end of the heterologous gene.

8. A DNA construct according to any preceding claim additionally comprising a splice acceptor, for example the rabbit b-globin splice acceptor, 5'(upstream) of the heterologous gene.

9. A DNA construct according to claim 8 in which the splice acceptor permits functional integration of the heterologous gene into an intron sequence.

10. A DNA construct according to any preceding claim additionally comprising a truncation/ cleavage/ transcription terminator sequence 5'(upstream) of X.

11. A DNA construct according to claim 10 in which the truncation/ cleavage/ transcription terminator sequence includes a splice acceptor and a polyadenylation signal.

12. A DNA construct according to Claim 10 or 11 omitting the IRES.

13. A DNA construct according to Claim 10 or 11 or 12 in which the transcription terminator is the Upstream Mouse Sequence or a poly A sequence, such as the SV40 polyadenylation signal.

14. A DNA construct according to any previous claim in which the heterologous gene codes for a selectable marker, such as antibiotic resistance, to facilitate selection of cells in which the heterologous gene has inserted into the host genome.

15. A DNA construct according to any previous claim further comprising a splice acceptor 5' to the IRES.

16. Use of a DNA construct according to any previous claim for inserting a heterologous gene into a host genome.

17. A method of inserting a heterologous gene into a target endogenous gene in a host cell genome comprising transforming the host cell with a vector comprising a DNA construct according to any of Claims 1-15.

18. A method of expressing a heterologous gene in a host cell comprising the steps:-

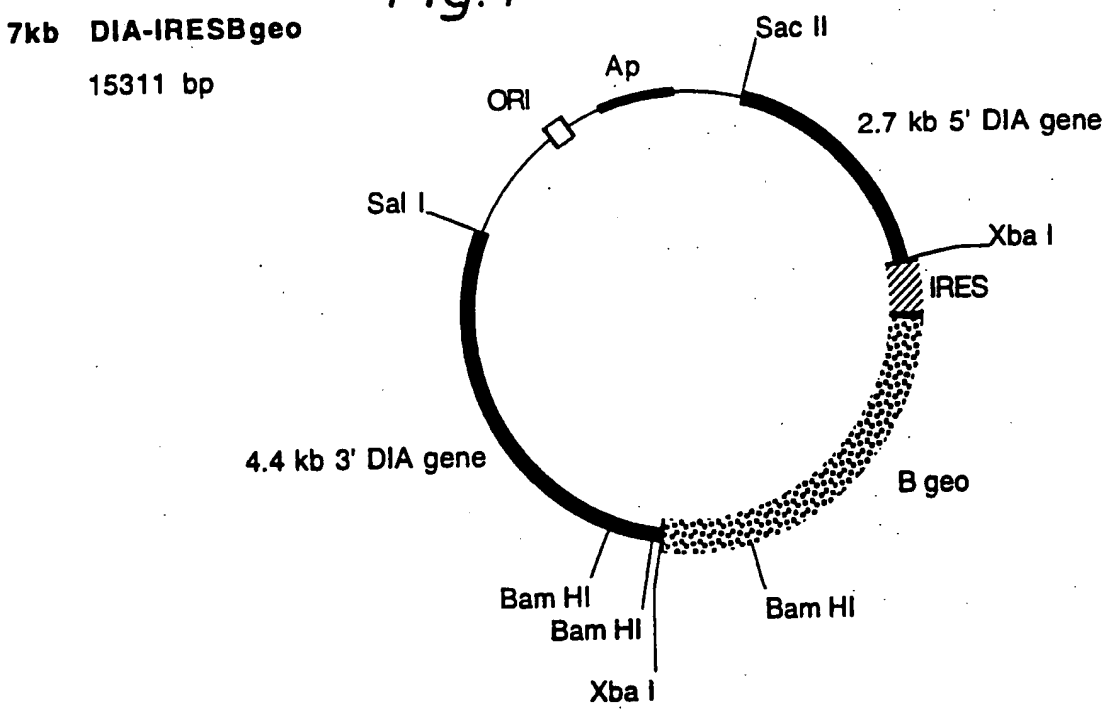
1. making a DNA construct according to any of Claims 1-15,
2. allowing the construct to undergo homologous recombination with or random integration into the host cell genome.

19. A cell or an animal comprising a heterologous gene inserted using a DNA construct according to any of Claims 1-15.

20. A descendant of a cell or an animal according to Claim 19, wherein the descendant has inherited the heterologous gene.

21. A vector containing a DNA construct according to any of Claims 1-15.

Fig. 1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Xba I

Fig. 2

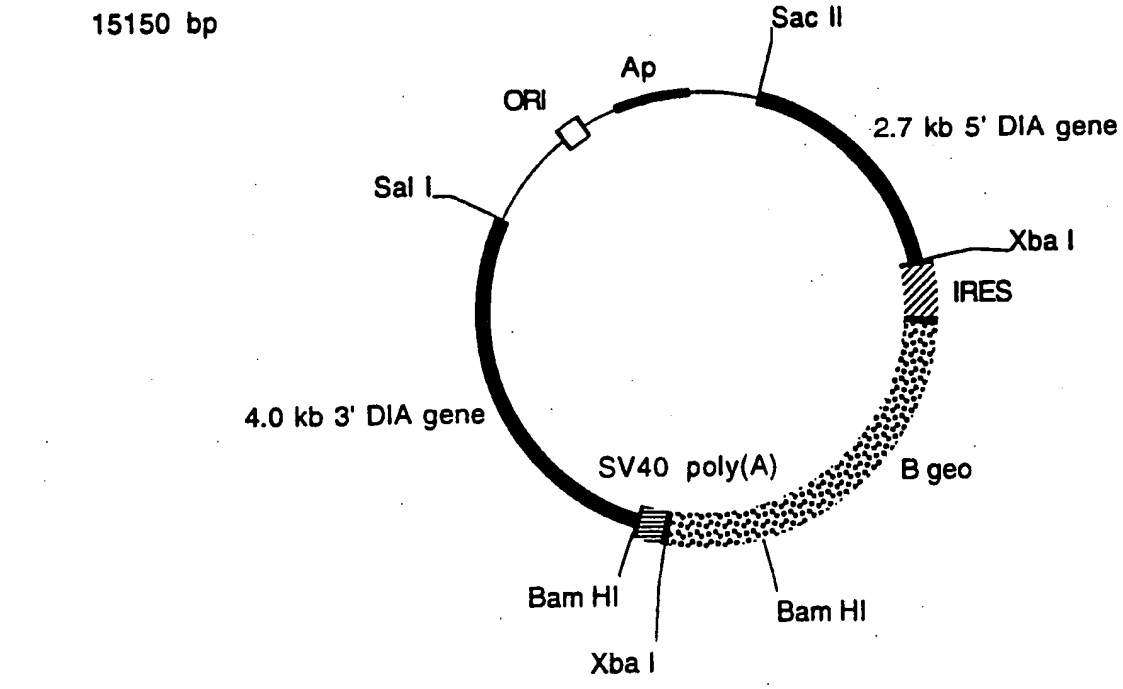
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Fig.3

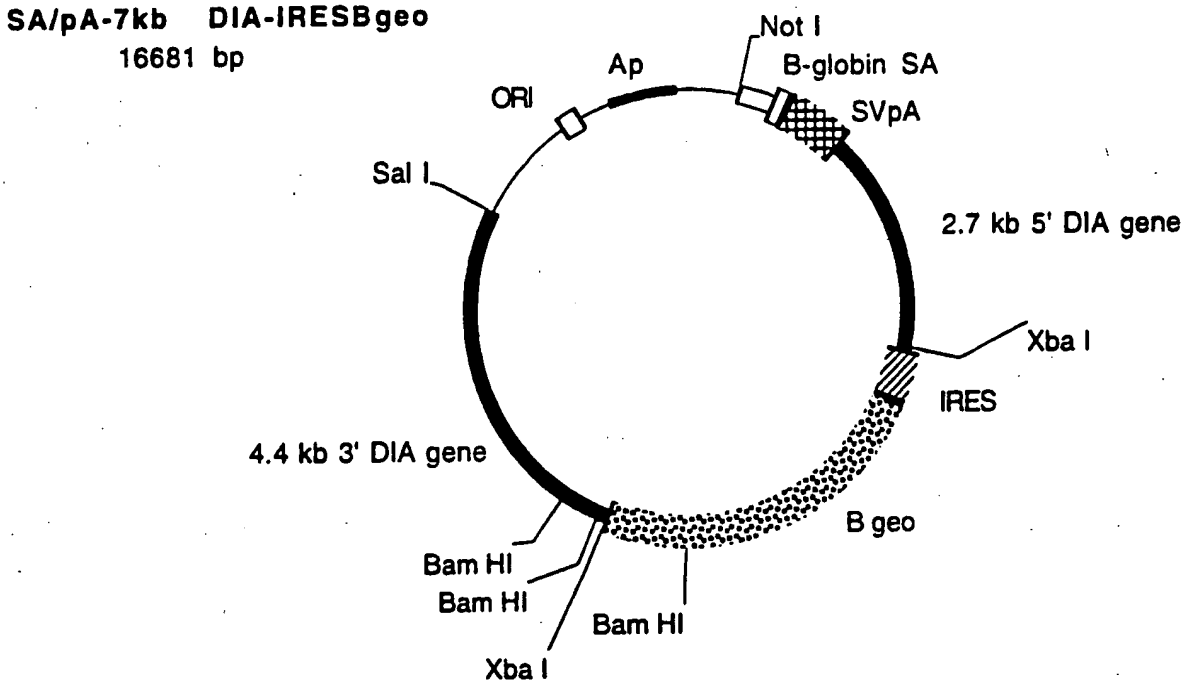
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Fig.4

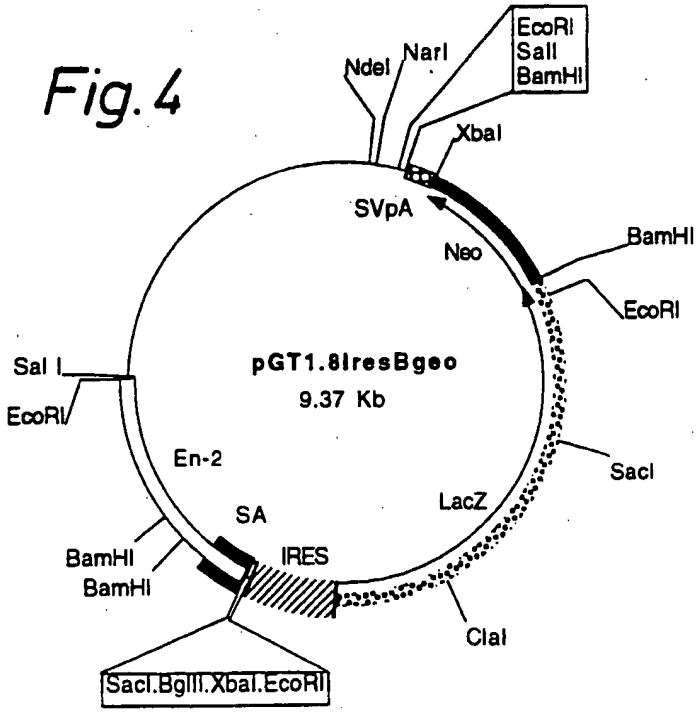
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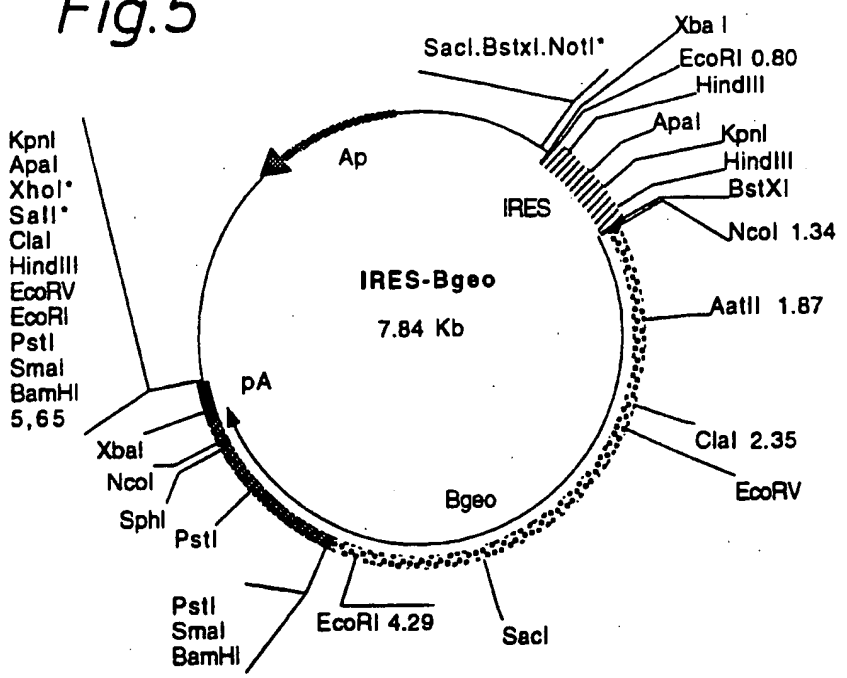
Fig.5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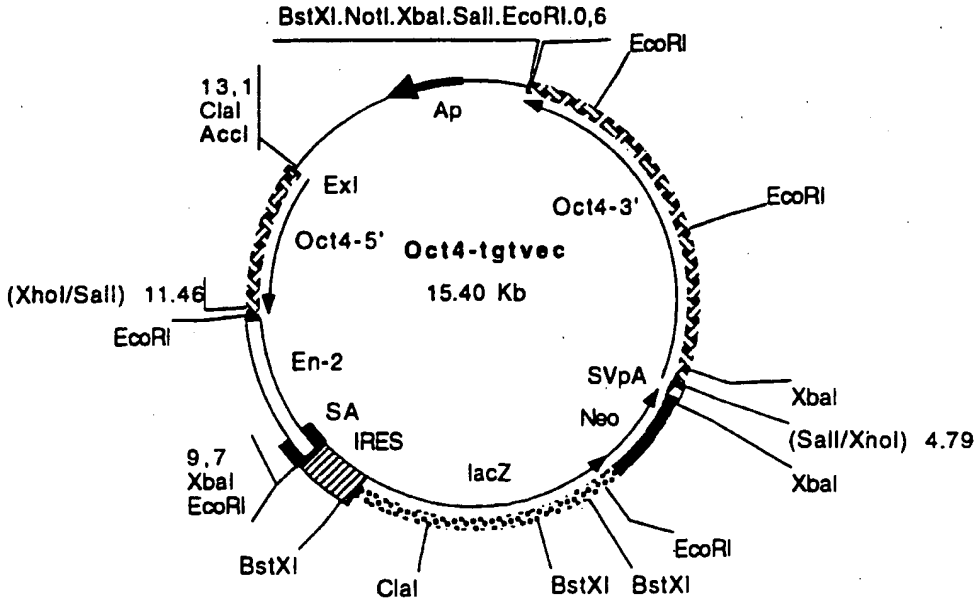
Bgeo

Fig.6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Neo
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Fig. 7

DICISTRONIC INITIATION OF TRANSLATION

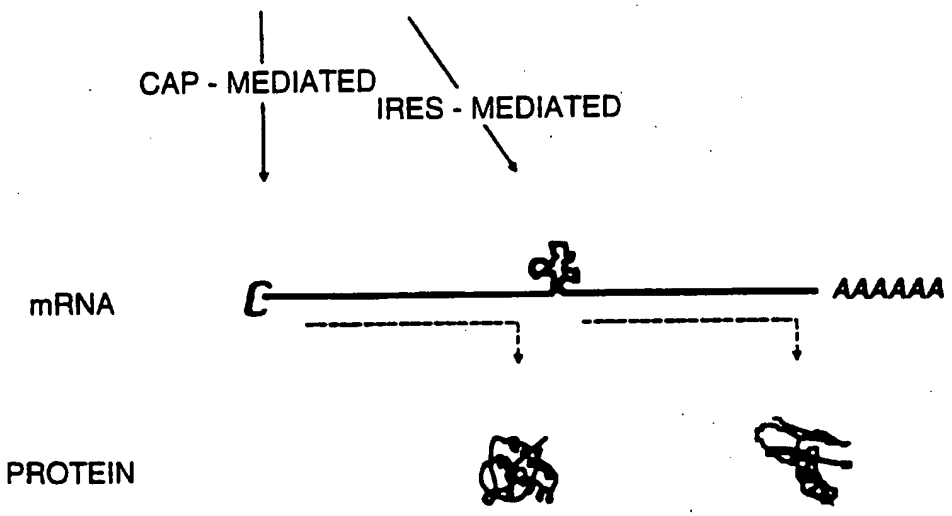
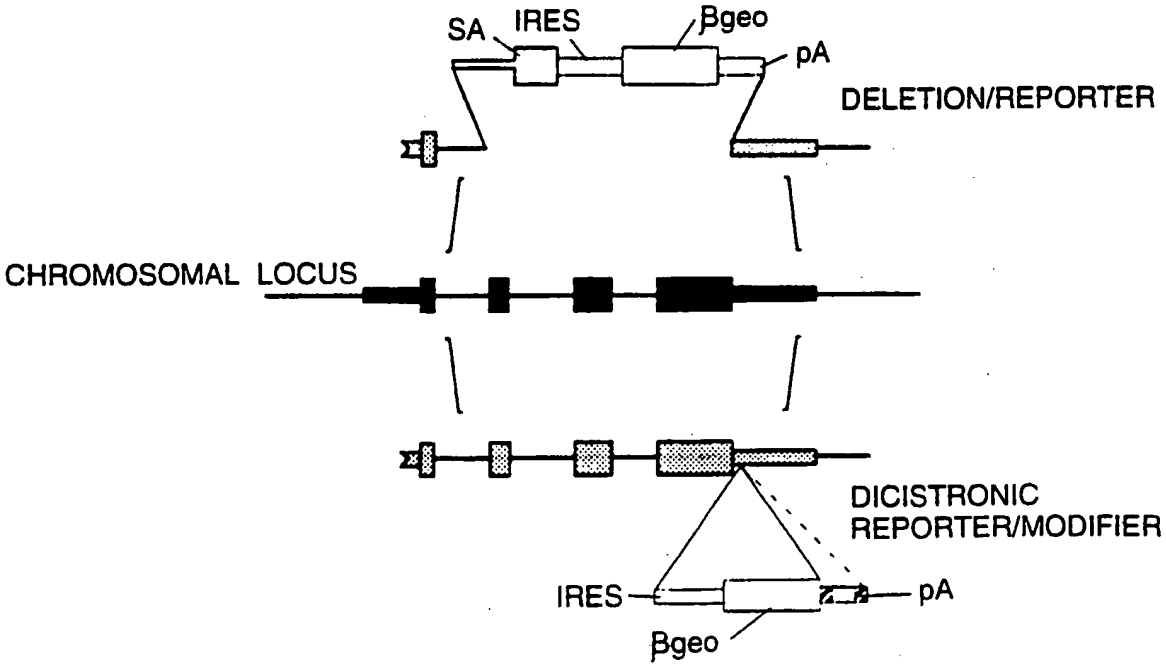
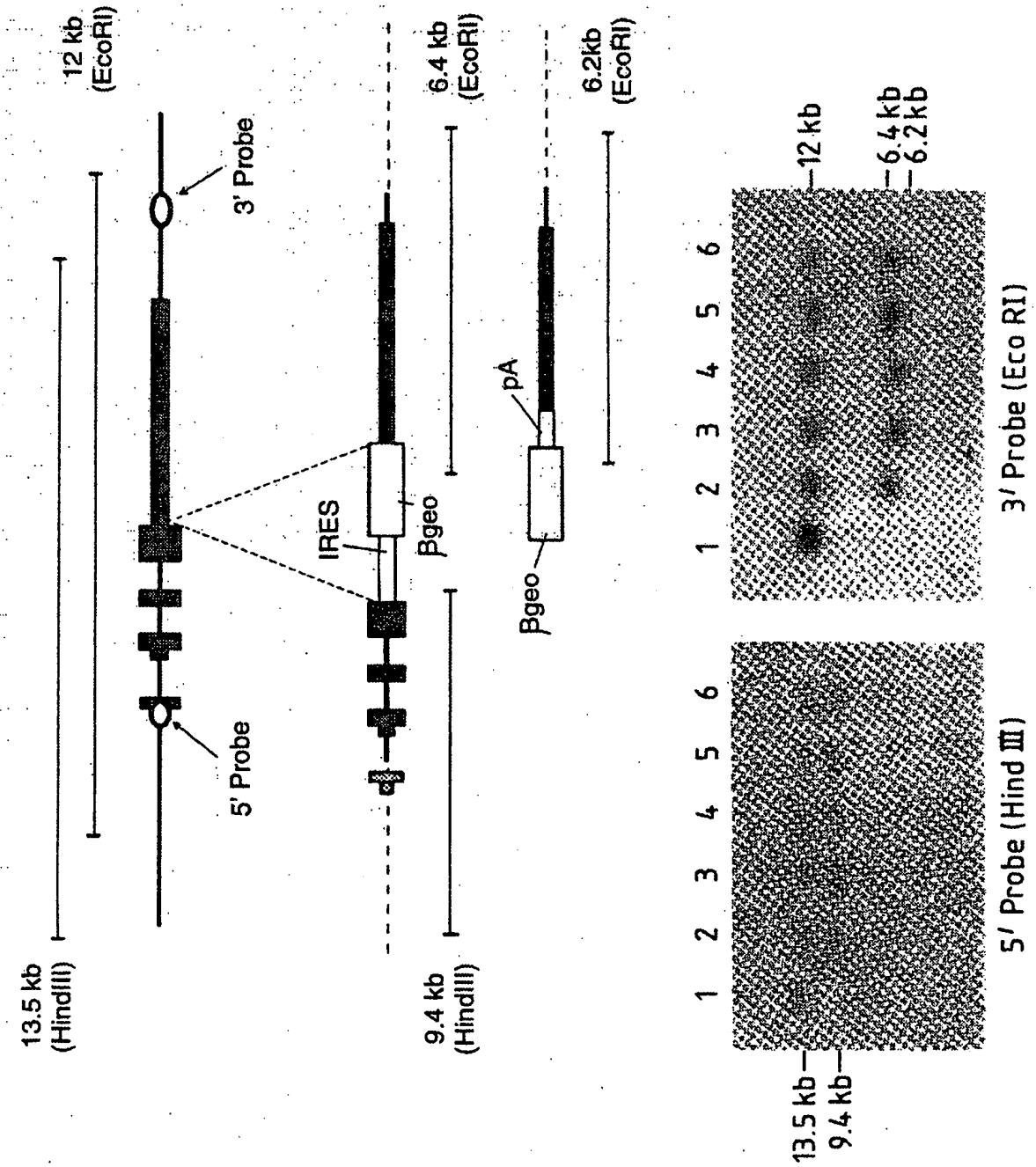


Fig. 8

TARGETING OPTIONS

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12 kb
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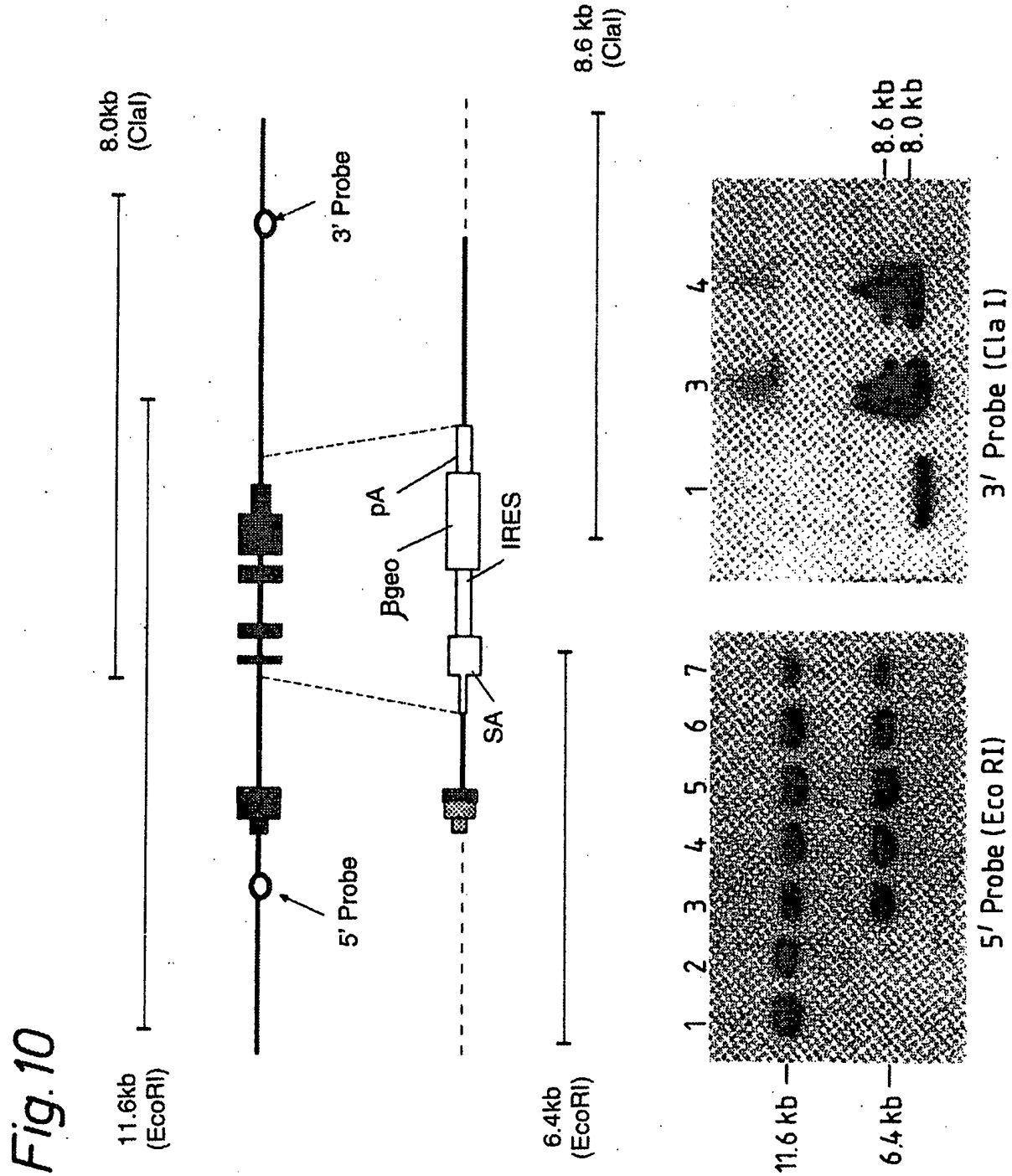
5' Probe (Hind III)

Fig. 9

Fig. 10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8.0 kb
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11.6 kb
6.4 kb
5' Probe (Eco RI)

Fig. 11.

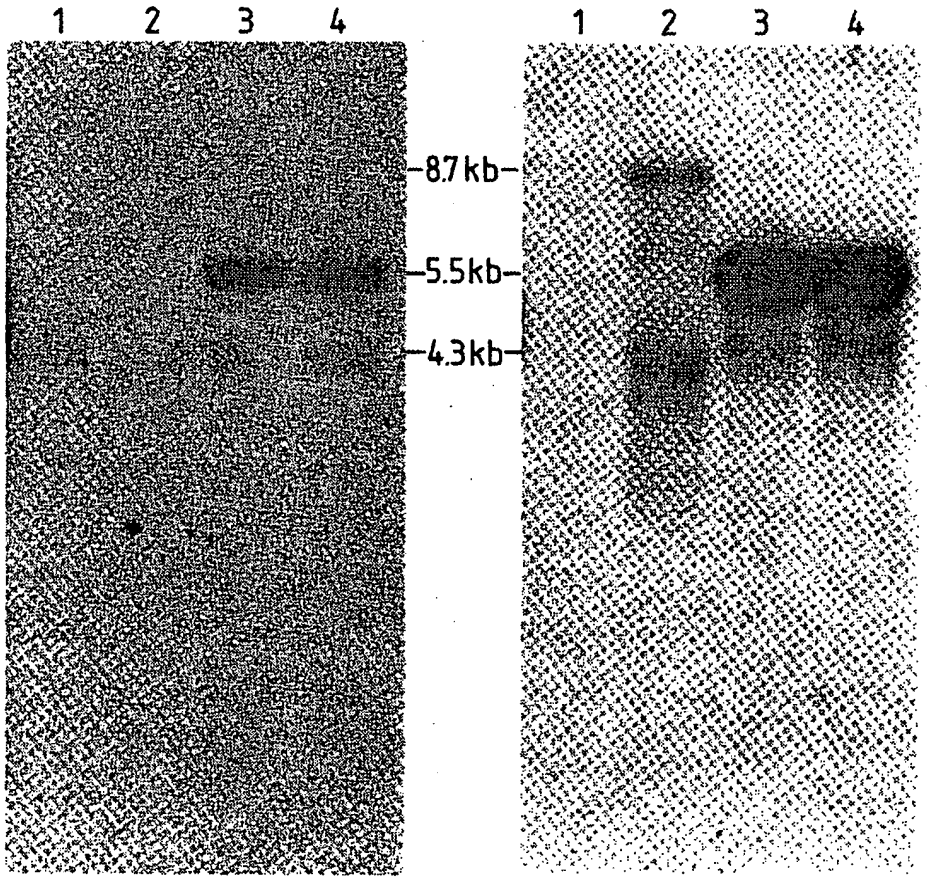


Fig. 12.

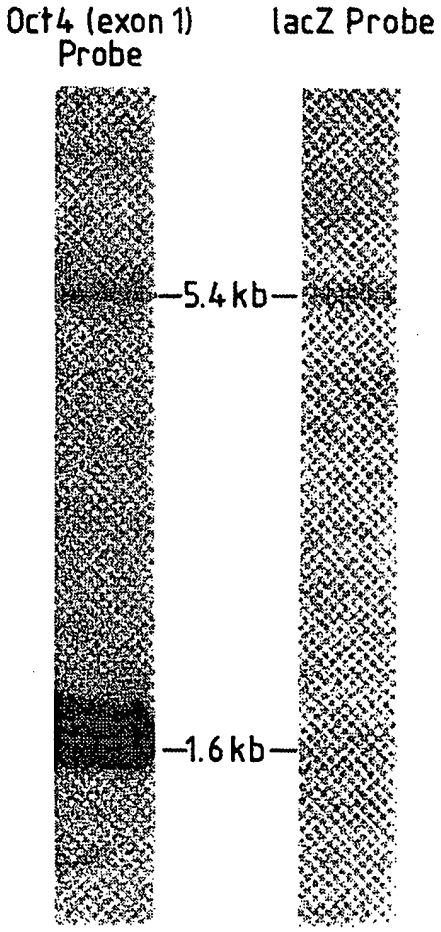
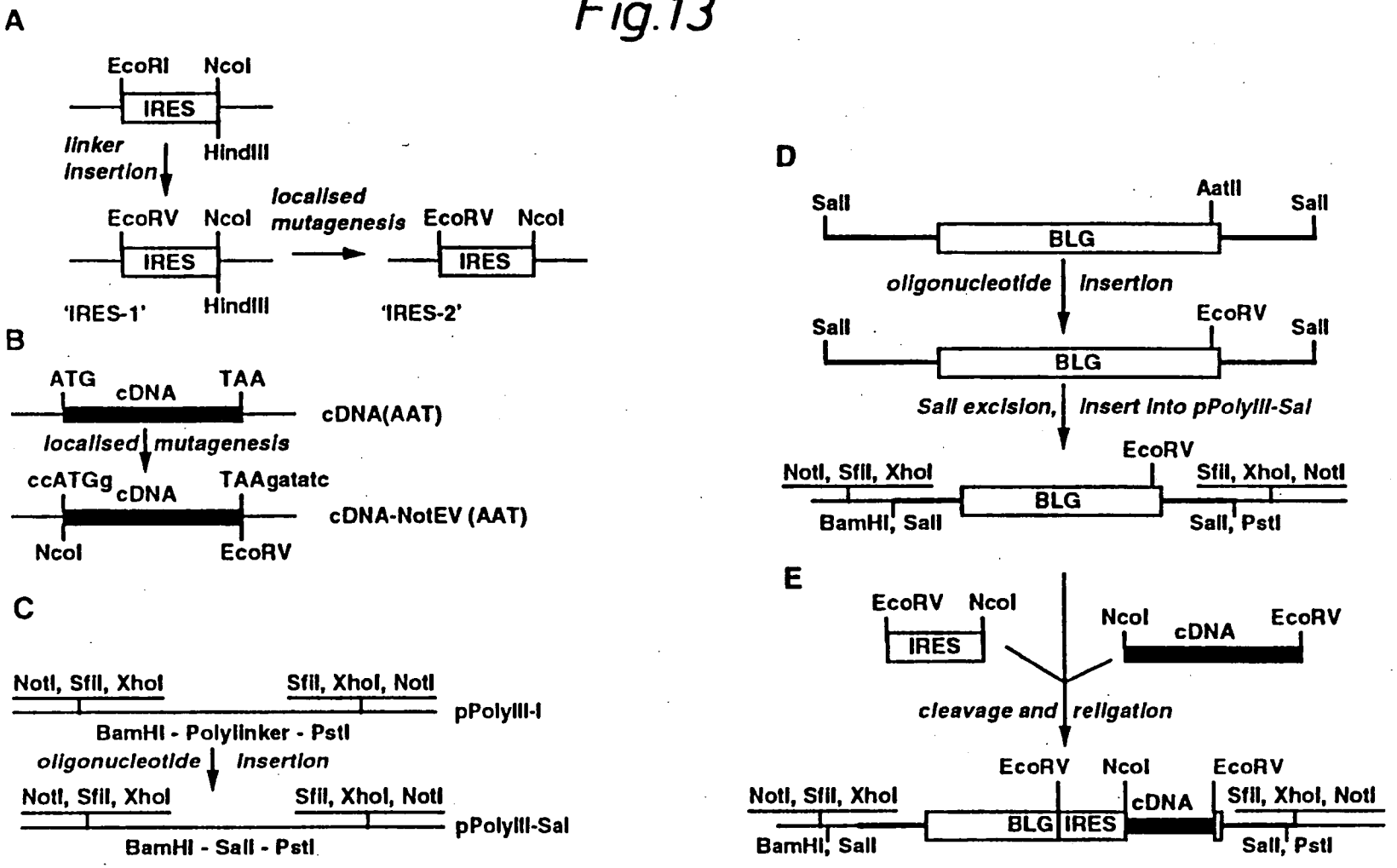


Fig.13


A
EcoRI
NcoI
IRES
HindIII
linker insertion
EcoRV
NcoI
IRES
HindIII
'IRES-1'
localised mutagenesis
EcoRV
NcoI
IRES
'IRES-2'
B
ATG
cDNA
TAA
cDNA(AAT)
localised mutagenesis
ccATGg
cDNA
TAAgatatc
cDNA-NotEV (AAT)
NcoI
EcoRV
C
NotI, SfiI, XhoI
SfiI, XhoI, NotI
pPolyIII-I
BamHI - Polylinker - PstI
oligonucleotide insertion
NotI, SfiI, XhoI
SfiI, XhoI, NotI
pPolyIII-Sal
BamHI - SalI - PstI
D
SalI
AatII
SalI
BLG
oligonucleotide insertion
SalI
EcoRV
SalI
BLG
SalI excision, insert into pPolyIII-Sal
EcoRV
NotI, SfiI, XhoI
SfiI, XhoI, NotI
BLG
BamHI, SalI
SalI, PstI
E
EcoRV
NcoI
IRES
NcoI
cDNA
EcoRV
cleavage and religation
EcoRV
NcoI
EcoRV
NotI, SfiI, XhoI
cDNA
SfiI, XhoI, NotI
BLG
IRES
BamHI, SalI
SalI, PstI

INTERNATIONAL SEARCH REPORT

Interna' l Application No
PCT/GB 94/00849

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/90 A01K67/027 C12N15/00 C12N5/10 C12N15/12
C12N15/19 C12N15/15

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A01K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	JOURNAL OF CELLULAR BIOCHEMISTRY vol. SUPPL, no. 18B , 21 January 1994 page 189 SMITH, G. ET AL. 'Targeted mutagenesis of the stem cell specific transcription factor oct-4' see the whole document	1
O,P, X	& Keystone symposium on stem cells Taos, USA January 31-February 7 1994 --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 August 1994	26 -08- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Chambonnet, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GENES AND DEVELOPMENT vol. 6, no. 6 , June 1992 pages 903 - 918 SKARNES, W.C. ET AL. 'A gene trap approach in mouse embryonic stem cells: the lacZ reporter is activated by splicing, reflects endogenous gene expression, and is mutagenic in mice' cited in the application	1
A	WO,A,93 07266 (IDAHO RESEARCH FOUNDATION) 15 April 1993 see the whole document	10-12
A	WO,A,90 11354 (INSTITUT PASTEUR) 4 October 1990 see the whole document	5
A	WO,A,92 20808 (CELL GENESYS, INC.) 26 November 1992 see the whole document	5

INTERNATIONAL SEARCH REPORT

I national application No.
PCT/GB94/00849

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Claims 16 to 18, as far as they are directed to a method of treatment of the human/animal body , have been searched based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/GB 94/00849

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9307266	15-04-93	AU-A-	2778992	03-05-93
WO-A-9011354	04-10-90	FR-A-	2646438	02-11-90
		EP-A-	0419621	03-04-91
		JP-T-	3504335	26-09-91
WO-A-9220808	26-11-92	AU-A-	2140292	30-12-92
		CA-A-	2092695	16-11-92
		EP-A-	0539573	05-05-93